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THE PRESERVATION OF YELLOW FEVER VIRUS*

By W. A. SAWYER, M.D., W. D. M. LLOYD, M.D., AND S. F. KITCHEN, M.D.

(From the International Health Division of the Rockefeller Foundation and The Rockefeller Institute for Medical Research, New York)

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In preparation for a comparative study of the characteristics of strains of yellow fever virus from West Africa and Brazil, we found it necessary to seek a reliable method of preserving the virus over a considerable period of time. The ideal method for our purposes would be one which would permit the sending of active virus in small sealed containers on sea voyages lasting over a month, and would also allow storage in the laboratory for several months without serious loss of virulence. Such a method was found among several processes previously applied to the preservation of other viruses, and it has now been in use long enough in connection with our experiments to show that it can be depended upon.

In the first work with experimental yellow fever in monkeys, Stokes, Bauer and Hudson (1) kept the virus alive for a time by passing it directly from animal to animal. They made inoculations by taking blood from the sick monkey, adding it to a citrate solution to prevent clotting, and injecting the mixture into a healthy animal. This method would have required an excessive number of monkeys to preserve even one strain of the virus during an extended study. The interval between successive inoculations had to be short for several reasons: the citrated blood could not be stored more than a few days without danger of losing the virus, the incubation period was usually brief, and the blood for transmission was drawn as a rule on the first day of illness. The difficulty was later diminished by these investigators by transferring the infection from monkey to monkey by means of mosquitoes, and thus lengthening to several weeks the time interval between successive monkeys in a series. This method is far from ideal, however, for the mosquitoes require much care and occasionally develop a high

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation. Through the courtesy of The Rockefeller Institute in New York laboratory facilities were made available.

mortality which might result in the loss of the yellow fever strain. The use of mosquitoes, moreover, necessitates special equipment and adds an extra hazard for the experimenters.

Sellards (2) succeeded in preserving yellow fever virus in frozen monkey liver for twelve days while transporting it from West Africa to England. Keeping tissues in a frozen state requires conditions difficult to arrange, especially on long voyages. The method, however, was one which promised to be highly useful, if tests proved it reliable for extensive periods, especially where the use of mosquitoes is impracticable.

The Yellow Fever Strains Used

For the purpose of comparing the values of different methods of preserving yellow fever virus we have brought together in tables the results of our observations with two strains which proved to be practically identical in their characteristics and very satisfactory for experimental work. Both were highly virulent and fairly constant in their effects. For one, the "French" strain ("F" in the tables), we are indebted to Dr. A. W. Sellards, who had brought it to America from West Africa via England. It had been transmitted to monkeys by Mathis, Sellards, and Laigret (3). The other is the "Asibi" strain ("A" in the tables), with which Stokes, Bauer, and Hudson did most of their work. It was originally obtained on the Gold Coast, West Africa, and was sent to us from Lagos, Nigeria, by Dr. Henry Beeuwkes, Director of the West African Yellow Fever Commission of the Rockefeller Foundation.

Sources of the Virus

In each instance the virus studied was preserved in the blood or the liver tissue of *Macacus rhesus* monkeys.

After an animal was inoculated, the rectal temperature was taken twice a day, and when the temperature rose to 40°C. for the first time, a specimen of blood was taken. The animal was anesthetized with ether and the blood was drawn from the heart.

The sick animals were allowed to die or were chloroformed when moribund. When needed as a source of virus, liver tissue was removed at necropsy with precautions for sterility. In all cases where an animal is reported as having died of yellow fever, the gross lesions of this disease were found on necropsy, and characteristic changes were seen in microscopic preparations of tissues, especially those of the liver. In a few instances the presence of tuberculosis or dysentery com-

plicated the picture and made it difficult to determine the onset and course of the yellow fever. Such cases were excluded from consideration in this study.

Subcutaneous or intraperitoneal injections of fresh first-day blood were almost invariably followed by the development of experimental yellow fever, even when small doses were used. As results with material which had been kept less than nine days are of little interest to this study, they have been omitted from the tables presented, except for the two partial titrations of fresh citrated blood of Monkeys 95 and 118 included in Table I. All injections of virus into monkeys in this study were intraperitoneal except in the case of the two titrations, in which they were subcutaneous. We have noticed no difference between the results following these two methods of inoculation.

The liver tissue proved less reliable for inoculation than the first-day blood. This is as would be expected when one considers that the virus diminishes rapidly in the blood after the first day or two of fever, and probably decreases in the liver during the latter days of the disease. The duration of the illness varies in the different cases and the concentration of virus in the liver at death probably varies also. Whatever the explanation, the liver tissue, though usually infectious, proved to be less consistently so than the first-day blood specimens.

Except when specially noted in the tables, no specimens which failed to infect are included unless the original fresh material was proven infectious by the inoculation of some other specimen derived from it. In a very few cases the inoculated monkeys had previously received material related to yellow fever, most often human blood two or three weeks old, and had shown no reaction. On account of the remote possibility that the animals had been protected by this material the letter "U," for "used," is placed after the number of each of these monkeys in the tables. The weights of monkeys are omitted from the tables, as moderate fluctuations seemed to play no noticeable part in determining the results of the tests. Specimens of monkey blood sent us from Lagos, Nigeria, are included in the tables and identified by specimen numbers preceded by the letter "S" instead of the numbers of the monkeys from which the virus was obtained.

The amounts of blood or liver are stated in the tables in terms of the fresh material. For example, 0.02 gm. of dried blood would be recorded as 0.1 cc., because blood loses about 81 per cent of its weight in drying. 2 cc. of glycerinated blood containing 1 cc. of actual blood would be recorded as 1.0 cc.

Incubation periods are shown in the tables as the interval between inoculation and the first day of fever; for example, if an animal was inoculated in the afternoon and its first fever occurred in the afternoon of the second day after inoculation, the interval would be recorded as 2.0 days, but if the first fever was in the morning of the third day, the period would be 2.5 days. The interval between inoculation and the time of death or recovery is similarly recorded. In the case of recovery the interval is measured to the last recorded temperature of 40°C. or over, and is followed by the letter "R." All deaths recorded were from yellow fever.

In order to limit as far as possible the number of monkeys needed for our investigations, most of the tests of preserved virus for infectivity were made as required by other experiments. This will explain the apparent lack of plan in some of the groups of observations presented.

TABLE I

Preservation of Yellow Fever Virus in Citrated, Clotted, or Glycerinated Blood

Method of preservation	Source, monkey and strain	Amount, blood, fresh	Age of specimen	Monkey inoculated	Interval in days to		Observation period	Result of test inoculation
					Fever	Death or recovery		
		cc.	*days*	*No.*			*days*	
Citration	95 A	0.6	0	99	1.5	5.0		
	95 A	0.08	0	100	2.0	4.5		
	95 A	0.005	0	101	10.0	11.0		
	118 F	0.9	1	129	6.0	7.0 R		Immune
	118 F	0.07	1	117	2.0	4.0		
	118 F	0.006	1	119	—*	8.5		
	18 F	0.6	22	31 U	—	—	24	Not tested
	32 F	3.5	22	29 U	—	—	24	Not tested
	66 F	2.0	30	112	—	—	30	Succumbed
	S 103 A	1.0	35	42	—	—	28	Not tested
	S 104 A	1.0	35	45	3.5	5.5		
	S 105 A	1.0	35	43	—	—	28	Not tested
Clotting	66 F	2.0	30	111	—	—	30	Succumbed
	S 106 A	1.0	35	46	21.0	24.5		
Glycerination	66 F	2.0	30	105 U	6.5	10.5		
	117 F	1.0	60	181	12.0	16.5		
	65 A	1.0	100	179	—	—	29	Succumbed

* No fever was observed before the temperature became subnormal, 6.5 days after inoculation.

Citrated and Clotted Blood

The most convenient method of making direct transfers of yellow fever virus from monkey to monkey was to prevent clotting by the addition of sodium citrate to the freshly drawn blood and to inject the mixture intraperitoneally or subcutaneously. On the day of the bleeding, or even the next day, the virus content of the citrated blood seemed to remain high, as illustrated by the first six items in Table I.

With longer storage the results became increasingly less dependable, and in the six tests of material from 22 to 35 days old there was only one successful inoculation (Table I).

In preparing the citrated specimens, from two to five parts of freshly drawn blood were mixed with one part of sterile 2 per cent sodium citrate solution, usually in 0.9 per cent sodium chloride or Locke's solution. The specimens were then stored in the refrigerator at from 1° to 4°C. The three citrated specimens received from Lagos (S 103, S 104, and S 105 in Table I) consisted of the blood of the same monkey plus an equal quantity of 2 per cent citrate solution. The specimen which proved to be infectious after the long journey from Lagos to New York was one of the two (S 103 and S 104) which had been kept in the ship's refrigerator. The other specimen of the same blood (S 105) had been stored at a higher temperature in the cool room.

If infectious blood is drawn and allowed to clot, it will retain its virulence for about the same length of time as if it had been citrated, as suggested in Table I by the one failure after 30 days in storage and the one success after 35 days. The infectious specimen of clotted blood (S 106) had come from Lagos and was from the same bleeding as the citrated specimens S 103, S 104, and S 105. It had been stored in the ship's cool room. It will be noticed that the incubation period in the case of the successful inoculation with clotted blood was 21 days, the longest in our experience.

Although the virus can be kept in clotted blood about as long as in citrated blood, citration is the more convenient method, for it is necessary to grind the clot with sand and physiological salt solution in a mortar before inoculation. If there is a sufficient amount of serum, this can be used without the clot.

In our experience it appears to be the exception for stored citrated or clotted yellow fever blood to retain its virulence as long as a month, and the method is obviously unsuited for prolonged storage. We made no experiments to determine whether the virus would persist longer in citrated blood under vaseline or oil, or in clotted blood kept continually frozen.

Glycerinated Blood or Liver

As glycerine had proved useful in the preservation of a number of viruses, we made preparations of blood and liver containing 50 per cent of glycerine and tested them for the presence of active virus after storage in a refrigerator at 2° to 6°C. The results are shown in Tables I and II.

The blood specimens were prepared by adding freshly drawn blood to an equal volume of glycerine in a test tube and shaking vigorously. The mixture was a thick fluid which usually clotted gradually in storage until it was of a firm, jelly-

like consistency and required grinding with sand to bring it into solution in physiological salt solution. The liver preparations were of two kinds. The second specimen of glycerinated liver listed in Table II consisted of pieces of liver tissue

TABLE II

Preservation of Yellow Fever Virus in Frozen, Glycerinated, or Dried Liver

Method of preservation	Source, monkey and strain	Amount, liver, fresh	Age of specimen	Monkey inoculated	Interval in days to		Observation period	Result of test inoculation
					Fever	Death or recovery		
		gm.	*days*	*No.*			*days*	
Freezing	65 A*	1+	12	89	—	—	48	Immune
	40 F	1+	15	66	6.5	9.0		
	18 F	1+	18	41	8.5	9.5 R		Not tested
	32 F	3+	20	40	5.5	8.5		
	97 A	1+	21	132	4.5	7.0		
	66 F	1.0	30	124	5.0	7.5		
	55 A*	1.0	30	106	—	—	30	Not tested
	66 F	1.0	100	191	—	—	30	Not tested
Glycerination	66 F	1.0	30	126	6.0	11.0		
	66 F	1.0	30	125	12.5	15.0 R		Immune
	117 F	1.0	60	187	9.0	12.0 R		Immune
	66 F	1+	100	193	1.5	5.0 R		Not tested
Drying	32 F	1.0	9	37	19.0	22.5		
	32 F	0.1	9	24 U	5.5	8.0		
	32 F	0.01	9	14 U	—	—	30	Not tested
	66 F	0.1	30	127	10.0	14.5		
	32 F	0.1	60	94	—	—	30	Not tested
	117 F‡	1.0	63	168 U	—	—	23	Succumbed
	32 F	1.0	100	146	7.0	8.0†		
	66 F	1.0	100	192	4.0	5.0		
	32 F	1.0	150	190	6.5	8.0		

* No control inoculation was made with the original liver tissue of this monkey to prove it infectious.

† Chloroformed early in the disease to secure material for pathological study.

‡ Tubed with calcium chloride.

dropped into 50 per cent glycerine in Locke's solution. The other specimens were prepared by grinding liver in an equal volume of Locke's solution, using sand, and then adding to the resulting suspension an equal volume of glycerine.

The results with glycerinated blood were distinctly better than with citrated or clotted blood, as will be seen in the tables. The samples of glycerinated blood

which had been kept 30 and 60 days were shown to contain active virus, but the incubation period for the 60-day specimen was prolonged. The 100-day specimen failed to infect and produced no immunity.

With the liver specimens we obtained infection in every instance, but recovery followed infection from one of the two 30-day specimens and from the 60-day and 100-day specimens.

The virus of yellow fever apparently can be preserved in 50 per cent glycerine for a longer time than in citrated or clotted blood. Infection has even been obtained with glycerinated material after storage for 100 days, but there is a distinct falling off in virulence with storage after 30 days. Stronger concentrations of glycerine have not been tried.

Frozen Liver

The method of preserving the virus in frozen liver proved to be reliable for periods of at least one month (Table II); but the single specimen tested after 100 days failed to infect. Pieces of liver in test tubes were placed in the freezing compartment of an ordinary household electric refrigerator. As the temperature in the compartment was $-12°C.$ the liver tissue was frozen hard.

On one occasion the refrigerating machinery did not function and the temperature in the compartment rose sufficiently to permit the liver tissue to thaw. As a result the specimens then in storage, the first four in Table II and also the seventh, were under refrigeration for two days without being frozen. The freezing method is simple and valuable when machinery is at hand for maintaining a constant temperature low enough to keep the tissues frozen hard.

Dried Blood or Liver

It was not until we tried drying blood or liver under vacuum in the frozen state and storing it in sealed tubes that we were able to preserve the virus of yellow fever for long periods.

The method was suggested to us by Dr. T. M. Rivers, who had found it satisfactory in the preservation of several viruses including vaccine virus and Virus III. What was essentially the same method had been used by Harris and Shackell (4) in 1911 in preserving rabies virus; and Rous (5) reported that in the same year the active virus of a transmissible sarcoma of fowls was stored successfully in dried and powdered tissue by Murphy. More recently the method of drying while in the frozen state was applied to the preservation of bacterial cultures by Swift (6).

Our usual procedure in preserving the yellow fever virus in dried blood commences with the drawing of 15 to 20 cc. of blood from the heart of a monkey under ether anesthesia on the first day of fever. The needle of the syringe is then thrust through several layers of gauze which cover a sterile, cylindrical glass evaporating dish 12 cm. in diameter and 6.5 cm. deep, and the blood is ejected. The thin layer of fluid blood on the bottom of the dish is then quickly frozen by setting the dish in a shallow pan containing alcohol and pieces of solid carbon dioxide.

At least an hour before the bleeding, a desiccator of the Hempel improved type with an internal diameter of about 15 cm. is packed in an ice-salt mixture in a rectangular metal pan. In the groove in the upper part of the desiccator is about 130 cc. of fresh concentrated sulphuric acid to absorb the moisture given off by the blood. In the bottom is enough glycerine to cover a porcelain platform and make a good contact with the evaporating dish.

When the blood in the evaporating dish is thoroughly frozen, the dish is lifted from the alcohol, the liquid is quickly wiped from the bottom, and the dish is placed in the desiccator. The cover of the desiccator is then given at least one complete turn to make sure that a good contact is made. An extremely small quantity of a suitable thick lubricant has been applied to the contact surfaces in advance. The desiccator is then evacuated with an electric air pump until the pressure has fallen to 1 or 2 mm. of mercury. The desiccator in its pan of ice and salt is put into a refrigerator to prevent rapid melting of the ice. From 16 to 20 hours later the vacuum is released and the desiccator is opened. The evaporating dish is taken out, wiped free of glycerine, and placed on a sheet of paper under a small glass-topped frame. The object of the frame is to prevent the distribution, by air currents, of infectious dust which might be inhaled by the operator. Rubber gloves are worn while the dried material is being tubed.

About ten plugged sterile test tubes measuring 12 by 200 mm. are numbered and weighed in advance. They are taken one by one, warmed slightly in the flame to prevent condensation of moisture, partially filled with the flakes of dried blood, flamed at the mouth, replugged, and again weighed. The gain in weight in grams represents the weight of the dried blood, which is approximately one-fifth of the weight of the fresh blood or of its volume in cubic centimeters. In the evaporating dish the dried blood is in the form of a thin, red, brittle wafer, with fissures running through it. It lies loose from the glass and can easily be broken into bits and transferred to the test tubes with forceps and narrow spatula. It is light and porous and can be readily crushed in a mortar to a smooth powder before it is tubed, but this probably increases the risk of accidental infection and ordinarily is of no advantage. After the weighing, the tops of the plugs are cut off with scissors and the remainder pushed down to the surface of the dried blood with a rod. To make certain of complete dryness, granular calcium chloride is poured in above the plugs before the tubes are sealed in the flame of a blast lamp. The tubes are stored in the refrigerator. The use of calcium chloride, although

perhaps advisable, is not necessary, since the oldest specimens tested were put up without it. The method of putting in the calcium chloride has been described by J. H. Brown (7).

When the dried blood is required for inoculation, the tube is opened by scratching with a file at the middle of the cotton plug and touching with a hot piece of glass or metal. The plug is carefully removed and a small amount of 2 per cent sodium citrate in physiological salt solution is put in to prevent scattering of dust. The contents are then emptied into a mortar, the adherent particles are scraped out of the tube with a platinum wire, and the material is ground up with additional fluid. Without the sodium citrate in the physiological salt solution the dissolved blood will clot in the mortar and make injection difficult.

Small quantities of blood, 1.0 cc. or 0.5 cc., are easily dried in a test tube or ampoule. The dry blood forms a small button which lies loose in the bottom of the container. The blood may be put up in ampoules with safety and with great simplicity of technique, but this advantage over the use of the evaporating dish and test tube is more than offset by the greater difficulty of getting the material out.

Where solid carbon dioxide ("dry ice") is not available the blood may be frozen in small amounts (1.0 or 0.5 cc.) in a number of test tubes in an ice-salt mixture before desiccation.

To preserve the virus in liver tissue, from 10 to 20 gm. of the fresh tissue is ground with sterile sand in a mortar without added fluid. The resulting paste is scraped into the glass evaporating dish and the sterile gauze cover is replaced and tied with string. Gentle tapping of the dish on a table top will complete the even distribution of the material over the bottom of the dish. The material is then frozen, dried, and sealed in tubes by the same technique as the one used for blood. As the total weight of the original material is known, the amount of liver tissue in each tube, in terms of fresh liver, is determined by dividing the original weight of the liver in proportion to the final weights of liver and sand in the several tubes. Inoculations are made with the material as in the case of blood, but physiological salt solution is used instead of citrate solution. The dried liver is soft, porous, and friable, and has a light greyish yellow color due to the presence of much fat.

Freezing is not absolutely necessary for the successful preservation of blood.

Specimen 157 A (Table III) was prepared in Lagos by the West African Yellow Fever Commission of the Rockefeller Foundation and was brought to us through the courtesy of Dr. Oskar Klotz of the Commission. Hudson and Klotz had demonstrated that infected monkey blood, desiccated in a vacuum in the presence of sulphuric acid or calcium chloride, at room temperature or in the icebox, remained infective as long as 38 days (8). In preparing Specimen 157 A the mixed blood of two monkeys was dried in a vacuum over calcium chloride and sealed

TABLE III

Preservation of Yellow Fever Virus in Dried Blood

Method of preservation	Source, monkey and strain	Amount, blood, fresh	Age of specimen	Monkey inoculated	Interval in days to		Observation period	Result of test inoculation
					Fever	Death or recovery		
		cc.	*days*	*No.*			*days*	
Drying	180 A*‡	2.5	12	202	—	—	32	Not tested
	180 A*‡	0.5	18	205	—	—	32	Not tested
	65 A	0.1	13	95	3.5	7.5		
	S 86 A¶	1.0	28	28	7.0	9.5		
	S 87 A¶	1.0	28	29	—	—	30	Not tested
	66 F	0.1	30	118	4.0	6.0		
	55 A	0.1	31	107	6.0	10.0		
	S 157 A¶	6+	32	103	3.5	7.5		
	117 F‡	0.3	42	144 U	—†	4.0		
	117 F‡	0.3	42	143 U	2.5	4.5		
	117 F‡	0.3	42	145 U	4.0	7.5		
	66 F	0.5	60	147 U	3.5	7.5		
	117 F‡	0.5	60	157 U	1.5	3.0		
	65 A	0.1	61	150	3.0	4.5		
	55 A	0.1	78	152	3.0	5.0		
	117 F‡	1.0	86	209	2.0	2.0‖		
	117 F‡	1.0	94	188 U	1.5	3.5		
	65 A	0.1	100	180	3.5	10.0		
	66 F	0.1	100	188	—	—	30	Succumbed
	55 A	0.1	101	176	9.0	11.0		
	55 A	0.01	101	177	—	—	37	Succumbed
	S 157 A§¶	0.1	102	173	3.5	5.0		
	55 A	1.0	109	151 U	3.0	5.5		
	65 A	1.0	127	210	—	—	30	Not tested
	55 A	1.0	154	235	4.0	6.5		
	S 157 A§¶	1.0	155	231	16.5	19.0		

* This specimen, when 6 days old, produced fever in a monkey in 2 days and death from yellow fever in 3.5 days.

† No fever observed.

‡ Tubed with calcium chloride. The other tubes were without it.

§ Re-tubed with calcium chloride when 43 days old.

‖ Killed to obtain material for study of early lesions.

¶ Dried without freezing.

TABLE IV

Groups of Specimens of the Same Material Preserved in Different Ways and for Varying Lengths of Time

Source, monkey and strain	Blood				Liver			
	Method of preservation	Amount, fresh	Age of specimen	Result. Yellow fever?	Method of preservation	Amount, fresh	Age of specimen	Result. Yellow fever?
		cc.	*days*			*gm.*	*days*	
32 F					Freezing	3+	20	+
					Drying	1.0	9	+
						0.1	9	+
						0.01	9	−
						0.1	60	−
						1.0	100	+
						1.0	150	+
55 A	Drying	0.1	31	+				
		0.1	78	+				
		0.1	101	+				
		0.01	101	−				
		1.0	109	+				
		1.0	154	+				
65 A	Glycerine	1.0	100	−				
	Drying	0.1	13	+				
		0.1	61	+				
		0.1	100	+				
		1.0	127	−				
66 F	Citration	2.0	30	−	Freezing	1.0	30	+
	Clotting	2.0	30	−		1.0	100	−
	Glycerine	2.0	30	+	Glycerine	1.0	30	+
	Drying	0.1	30	+		1.0	30	+ R
		0.5	60	+		1+	100	+ R
		0.1	100	−	Drying	0.1	30	+
						1.0	100	+
117 F	Glycerine	1.0	60	+	Glycerine	1.0	60	+ R
	Drying	0.3	42	+	Drying	1.0	63	−
		0.5	60	+				
		1.0	86	+				
		1.0	94	+				

in a glass tube. It was transported from Africa to America at room temperature. A part was used in a successful inoculation when the material was 32 days old. The remainder was retubed with calcium chloride and was found to be highly virulent when 102 days old (Table III). It infected also after 155 days, but the incubation period was prolonged. The dried blood of this specimen consisted of small black glassy scales in striking contrast to the soft porous red pieces of the blood which was frozen before being dried. Specimens 86 and 87, from Lagos, were also dried without preliminary freezing. One of the two was found to be infectious when 28 days old (Table III), but the other was inert.

The specimens of blood and liver which had been frozen and dried seldom failed to produce infection. Two of the three specimens of blood which were tested when 100 days old were found to be virulent when injected in amounts as small as the equivalent of 0.1 cc. of fresh blood. One of these two failed to infect at 127 days. The other was tested again when 154 days old, in a dosage equivalent to 1.0 cc. and produced yellow fever after an incubation period of only four days. Tests after longer periods have not yet been made.

The dried liver gave similar results, but the observations were fewer (Table II). Our oldest specimen (from Monkey 32) failed to infect when 60 days old when a dose equivalent to 0.1 gm. of fresh liver was injected, but infected after 100 days and after 150 days when the equivalent of 1.0 gm. was injected. Another specimen 100 days old was tested and found to be infectious.

The process of freezing and drying will preserve bacteria as well as the virus of yellow fever, and one would expect to get bacterial infections occasionally from the use of dried liver, since pathogenic organisms frequently invade the liver late in the disease. Such infections would occur very seldom when dried blood is used. As the result of inoculation with a specimen of fresh yellow fever liver we lost two monkeys from a streptococcus peritonitis, and inoculation with another specimen, which had been sent us under refrigeration, caused three animals to develop tuberculous peritonitis.

Comparison of Methods

A close comparison of the value of the methods can only be made by submitting the same original material to the several processes. One specimen of fresh blood or liver may contain many times as much virus as another. We have, therefore, brought together in groups in Table IV the specimens derived from each of several lots of blood or liver. Each of these specimens appears also in one of the previous tables, where additional data are given.

CONCLUSIONS

1. The virus of yellow fever may be preserved for at least 154 days in the blood or liver tissue of infected monkeys if the material is dried

in a vacuum while in the frozen state and kept in the refrigator in sealed glass containers. A gradual diminution of virulence is noticeable in the older specimens.

2. If infectious blood is dried in a vacuum at room temperature, instead of in the frozen state, and is stored in sealed containers in the refrigerator, the virus may survive as long as 155 days.

3. The virus may be preserved for at least 30 days in liver kept continuously frozen.

4. Storage of blood or liver in 50 per cent glycerine in the refrigerator will usually keep the virus alive for 60 days and may do so for 100 days, but with the injection of the older material there is a marked tendency toward lengthening of the incubation period and increase in the number of recoveries.

5. Yellow fever virus in citrated or clotted blood, when kept in the refrigerator, dies out rapidly.

6. In our experience the most satisfactory method of preserving strains of yellow fever virus in the laboratory consists of freezing and drying blood taken from a monkey on the first day of an attack of experimental yellow fever and storing the dry material in sealed glass tubes in a cold place.

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A COMPARISON OF THE MANNER OF EXCRETION OF NEUTRAL RED AND PHENOL RED BY THE FROG'S KIDNEY*

By JEAN OLIVER, M.D., and ESHREF SHEVKY, Ph.D.

(From the Department of Pathology of Stanford University Medical School, San Francisco, California)

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Since the beginning of modern physiological experimentation on the function of the kidney, the study of the manner of its excretion of dyes has proved a fruitful method of attack on the problem of the activity of its complex elements. The literature covering the subject matter of these studies has grown to vast proportions, but needs no review at this time as it has been frequently and thoroughly discussed.

Our interest in the manner of excretion of two dyes, phenol red and neutral red, arose in the course of an investigation of the problems of experimental nephritis which we had produced in frogs and which we studied by means of the newer methods devised by physiologists for the examination of their problems. Among these methods is that of perfusion of the kidney.

The perfusion of the amphibian kidney offers many advantages over similar procedure with mammals. Among the first to use the method extensively were the English observers, Bainbridge and his coworkers (1) and more recently Höber and his pupils (2) have published a long series of studies on the normal function of the frog's kidney. Among their many observations they found evidence indicating that the dye cyanol was not excreted by the tubule but by the glomerulus.

Our original problem was the testing of damaged kidneys, and among other methods that of perfusion was used. One of the reagents

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employed was phenol red. As another line of attack, vital staining with neutral red was employed. It soon became evident that the kidney handled the two dyes in different ways and in view of the conflict in opinion of various workers on the subject it was thought worth while to extend our investigation to a more detailed study of the manner in which the two dyes are excreted by the normal kidney. Our findings seem to warrant consideration for themselves as distinct from their bearing on the more specific problem of the abnormal kidney. They are, therefore, presented separately.

Methods

The method of perfusion which we have used is essentially that devised by Barkan, Broemser and Hahn (3) and elaborated by Höber.

Large bull frogs were used (*R. catesbiana*), averaging 800 gm. in weight, as this allows the collection of considerable amounts of urine, at times as high as 20 cc. or more per hour, and a correspondingly accurate determination of rates of excretion of various substances. The perfusion fluid was that of Barkan, Broemser and Hahn, consisting of a modified Locke's solution buffered to a pH of 7.4 and oxygenated with the proper mixture of CO_2 and O by means of their apparatus. Glycocol in a concentration of .52 per cent was added according to the recommendation of Höber (4) and glucose in a concentration of .05 per cent. The frogs were pithed, the viscera with the exception of the liver and heart, lungs and kidneys removed, with ligation of all severed vessels, and canulae introduced into the anterior abdominal vein—thus supplying the renal portal venous system, —and into the aorta just below the union of the two thoracic branches. In order to restrict the perfusion as much as possible to the kidneys the aorta was then tied below the renal arteries, the large veins to the legs ligated and the vena cava cut to allow the escape of the perfusion fluid which had passed through the kidney. The perfusion pressure was always kept as low as compatible with a free flow of fluid through the vessels, as a rule the venous pressure being 20.0 cm. of water, the arterial 40.0 cm. In both ureters were placed collecting canulae and the urine was removed in 15 minute periods.

Certain routine data were collected in all experiments. The rate of water excretion in cubic centimeters per hour was recorded and the presence or absence of sugar was tested with Benedict's solution. The total electrolyte content was followed by means of a Christiansen ionometer, giving the relative concentration of salts which was expressed as a percentage of NaCl. The concentration of dye in the urine was determined with a Duboscq colorimeter and a known standard. In our findings only one typical example is given, but for each experiment sufficient confirmatory experiments were performed to leave no doubt of the result.

EXPERIMENTAL

The excretion of phenol red when supplied to the tubules alone was compared with its excretion when supplied only to the glomeruli.

In these experiments phenol red in a concentration of 20 mg. per 1000 cc. of perfusion fluid was supplied to the tubules by way of the vein, while Locke's

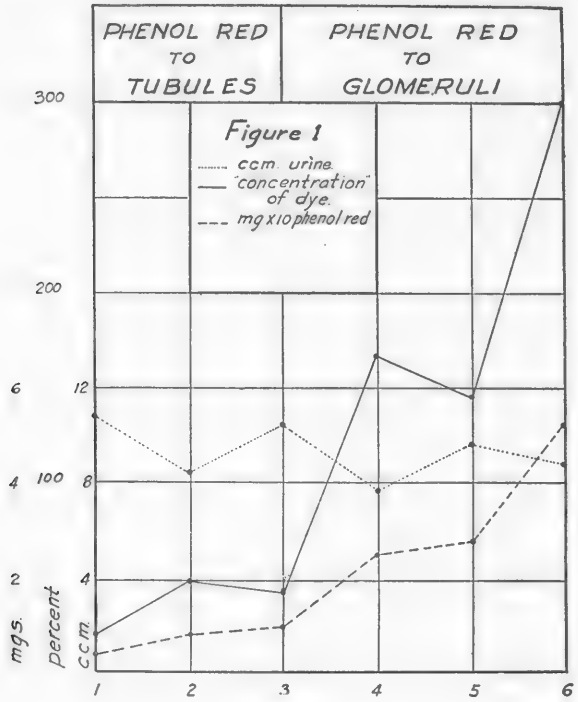

FIG. 1

solution free of dye was at the same time perfused through the glomerular tufts by way of the aorta and renal arteries. The pressure in the latter was about 45 cc. of water as compared to only 20 cc. in the venous tubular system. Before the addition of the dye the kidneys were perfused as routine with clear Locke's solution through both systems for two 15 minute periods at least to be sure that they were working in a normal manner. This was assumed to be the case, in accordance with Höber's findings (2), when the urine was free of sugar and its salt

content was not more than 50 per cent of that of the perfusion fluid. After the introduction of the dye, the 15 minute collections were tested in the routine way and the presence of dye noted and its concentration determined. The results are shown in Fig. 1.

During the preliminary periods the urine was sugar free and its salt content 40 per cent of the perfusion fluid. On the introduction of the phenol red into the fluid which went to the tubules no significant change was noted in any of its constituents. The presence of the dye was just perceptible to the eye and on determination was found to be about 50 per cent of that in the perfusion fluid the rate of excretion being .08 mg. per hour. After 3 periods the manner of dye administration was reversed, the concentration previously given to the tubule being led to the glomerular circulation by way of the aorta and renal arteries, while the tubular system of capillaries received clear Locke's solution by way of the renal portal vein. In other experiments dye was added to the glomerular fluid as described above, while the tubular system continued to receive the dye containing Locke's solution with no significant difference in result. The effect of this change in the method of administration of the dye is shown in the 4th period of Fig. 1. The excretion of water, sugar and salt remained practically unaltered but there was a sudden and immediate increase in the dye excretion from a previous level of 50 per cent of the perfusion fluid to 300 per cent. Expressed as rates of excretion, the rise was from .08 mg. per hour to .52 mg. per hour, or a six fold increase. In this particular experiment the increase in the amount of dye excreted by the glomeruli as contrasted to the amount put out by the tubules was not as large as that usually obtained. A summary of nine experiments gave an average increase of twenty-six times, the range of variation being from six to fifty fold.

Evidently phenol red when supplied to the glomeruli of the frog's kidney is excreted at a rate which may be as much as 50 times as great as when it is supplied to the tubules. Some dye, a small amount indeed, is excreted however when the supply is to the tubules alone, and this finding might be interpreted in one of two ways. Either this slight excretion is through that part of the kidney directly supplied by the renal portal system, that is through the tubules, or the fluid from the tubular venous capillaries passes over into the glomerular circulation in small amounts and the dye contained in it is excreted by the glomerulus.

We see no way to devise an experiment that will furnish an absolute control to the first possibility. It is a simple matter, however, to show that the second is not only possible but highly probable, for it has been demonstrated by others that fluid certainly does pass from the tubular to the glomerular circulation, especially if the proper rela-

tions between the pressures in the two systems are not maintained. The following experiment illustrates this point (Fig. 2).

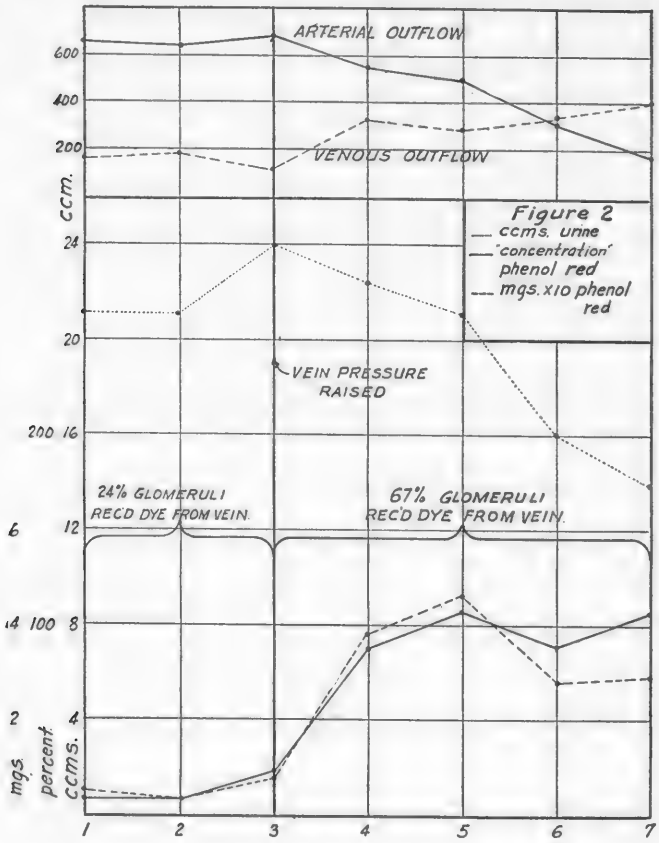

FIG. 2

After two preliminary periods in which the kidney functioned normally, 20 mg. of phenol red and 2 cc. of India ink suspension were added to 1000 cc. of Locke's solution in the bottle supplying the venous system, while the arterial bottle which supplied the glomeruli contained plain Locke's solution. The height of the two bottles was the usual one, 20 cm. for the venous tubular system and 45 cm. for the arterial glomerular bed. The out-flow from the two bottles through the two

systems is shown in the graph, the arterial flow being greater than the venous flow. which is the normal relation for perfusion by the method.

As will be seen from the graph, an average concentration of dye equal to only 10 per cent of that of the perfusion fluid was observed, while these conditions prevailed (Periods 1, 2 and 3). This equals a rate of excretion of .04 mg. per hour. The left kidney was then quickly removed with ligation of its vessels so that there would be no leakage during perfusion and fixed in 10 per cent formalin.

The pressure in the venous capillaries was now increased by 10 cm. Thereupon the flow through the venous capillaries increased considerably and though the rate of water excretion remained approximately the same, the concentration of dye rose to 90 and then 110 per cent of that of the perfusion fluid, or a rate of .45 mg. per hour (Periods 4 and 5). This figure is 10 times the rate of the preceding periods. The right kidney was then removed and fixed. Sections of the two kidneys were prepared and examined. The number of glomeruli which contained carbon particles, that is, which had received fluid and dye from the tubular circulation, in the case of the left kidney which had excreted .04 mg. per hour of the phenol red was 24 per cent of the 150 counted. In the right kidney where the rate of dye excretion had risen to .45 mg. per hour, 67 per cent of the glomeruli counted contained carbon as evidence of an increased flow from the tubular venous systems.

This experiment leaves no doubt that some dye reaches the glomeruli from the venous circulation, that the amount of such dye increases as the pressure on the fluid in the latter system is increased, and that concomitantly with this increased passage of dye to the glomeruli there is an increased rate of dye excretion by the kidney. We therefore believe it likely that the small amount of dye which was observed to be excreted in our first experiment during the period of tubular perfusion was evidence not of tubular excretion of the dye but of an unavoidable imperfection in the method of experiment. Some leakage from the tubular to the glomerular system cannot be avoided so that glomeruli receive the dye, and as the second part of the first experiment shows, it is readily excreted through them.

The excretion of neutral red when supplied to the glomeruli alone was compared with its excretion when supplied only to the tubules.

Before detailing the results of our experiments on the excretion of neutral red by perfused kidneys, it is necessary to test by experiment the statement of certain investigators who claim that neutral red is not excreted by the kidney of the living frog.

10 cc. of saline containing .5 mg. of neutral red was injected into the dorsal lymph sac of a frog whose bladder had been emptied by a catheter. The animal was kept in a glass jar where any urine passed might be recovered. After 60 minutes the bladder was again emptied. 2 cc. of urine was obtained containing .05 mg. of the dye. It follows that 10 per cent of the amount injected was recovered.

The same method of experiment was then applied to rabbits. Five mg. of neutral red was given intravenously in 20 cc. of 3 per cent NaCl solution. After

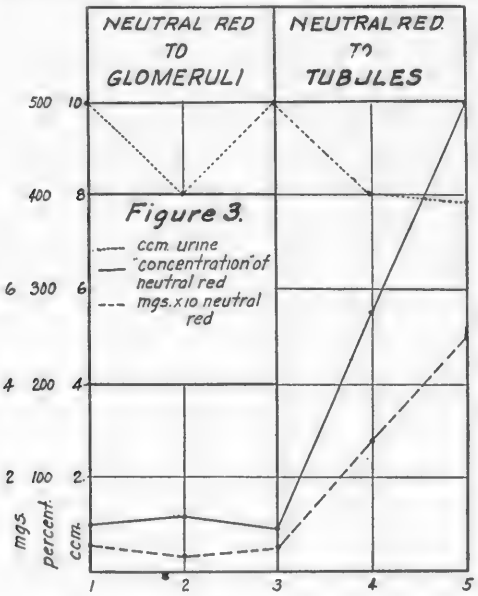

FIG. 3

1 hour the animal was catheterized again. One rabbit excreted 16 per cent, another 12 per cent of the dye. The average excretion of 8 more animals was 21 per cent with a range from 14 to 31 per cent. As there seems no doubt that neutral red is excreted by both the amphibian and mammalian kidney, the manner of its excretion was further examined by the method of perfusion.

The first perfusion experiment to be described is similar in its method to the first one described with phenol red. Other experiments relating to vital staining of the kidney with the neutral red had led us to suspect that it might be handled in a different manner than is phenol red. For this reason this dye was first introduced into the glomerular system by way of the artery and after a certain number of

periods was then supplied to the tubular apparatus by way of the renal portal vein. Fig. 3 shows the result. During Periods 1, 2 and 3 in which the dye reached the glomeruli in large amount, only small amounts appear in the urine, averaging about 50 per cent of the concentration in the perfusion fluid, an average rate of .06 mg. per hour. The method of administration was then reversed, the neutral red now going direct to the tubules by the vein and the glomeruli receiving clear Locke's from the arterial bottle. The concentration of dye in the urine rose in the next (4th) period to 260 per cent, a rate of .24 mg. per hour and increased in the 5th period to a figure of 500 per cent, 5½ times as concentrated as the perfusion fluid, a rate of .50 mg. per hour.

The rate of excretion of neutral red with tubular perfusion of the dye was therefore 8 times that obtained when the dye was administered to the glomeruli. Summarizing twelve experiments shows that the average increase in the rate of excretion of the neutral red by tubules as contrasted to the glomerular output was thirty-five times, the range of variation being from eight to sixty fold.

If it be a fact, as the above experiments would seem to indicate that the excretion of the two dyes is by different mechanisms, phenol red being excreted chiefly by the glomerular apparatus and neutral red by the tubular system, then it should be possible to devise crucial experiments which will confirm indirectly the results described above that were obtained by direct observation.

If, for instance, the above hypothesis be true, it should be possible to administer both dyes to kidney at the same time in such a manner that no dye in any considerable amount appears in the urine. The following experiment demonstrates that this can be done.

The perfusion experiments previously described were repeated, but to the glomerular circulation neutral red in a concentration of 12.5 mg. per 1000 cc. was supplied while a similar concentration of phenol red was led to the tubule capillary system. Fig. 4 illustrates the results. Although both perfusion bottles contained highly colored fluid, the urine in the ureteral canulae was practically colorless to the unaided eye. The urine sample for each period was divided into separate portions, one being acidified to produce the red form of neutral red and another alkalinized to show the yellow form of phenol red. In the first sample no phenol red could be demonstrated. Neutral red in an amount averaging 15 per cent of the concentration in the perfusion fluid was present.

Clear Locke's solution, free of either dye, was now supplied to the tubular apparatus by the vein, and phenol red led to the glomeruli by the arteries. In the next period (2) 570 per cent of the concentration in the perfusion fluid appeared

in the urine, a rate of 1.5 mg. per hour. In the next period about one half as much was excreted.[1]

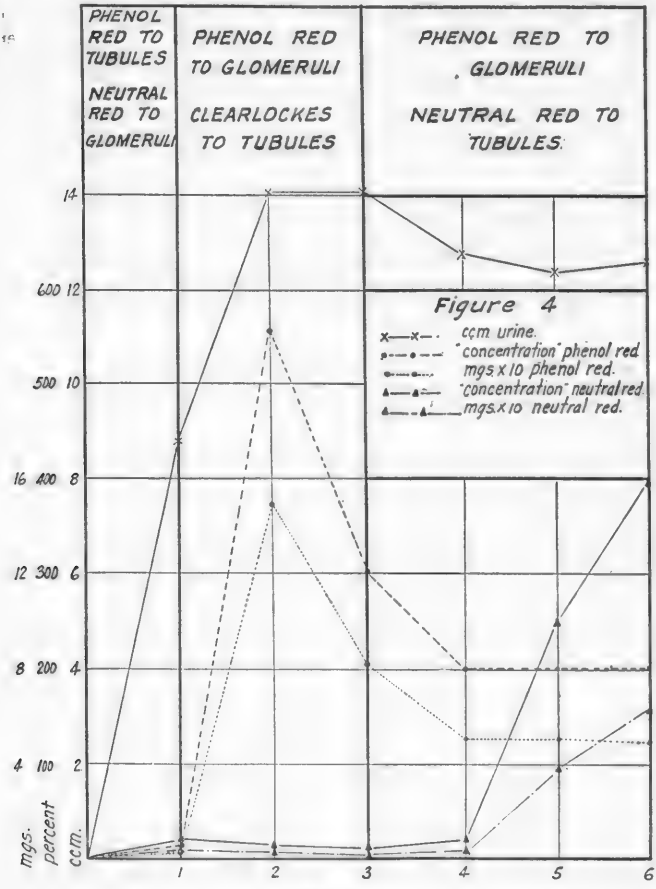

FIG. 4

Neutral red was now added in its original concentration to the fluid supplying the tubular system, the phenol red still being present in the glomerular supply.

[1] As we will show in a later study, the dye is excreted in greatest amount in the first period and then falls to a fairly constant level.

By separating the urine as in the first periods, making one acid and the other alkaline, an approximate estimation of the amount of each dye could be made. Exact color matches in the colorimeter were impossible but rough estimations could be made. In the 5th period the two dyes were present in about equal concentration, 200 per cent for phenol red and 250 per cent for neutral red. In the 6th period the latter had increased to 400 per cent, the phenol red remaining approximately 200 per cent of the concentration existing in the perfusion fluid. The final rates of excretion for the two dyes was .5 mg. per hour for neutral red and .45 mg. per hour for phenol red.

It will be seen that the two dyes, when supplied to the kidney the "wrong way to" were not excreted in any significant amount. When the method of administration was corrected to correspond to the normal manner of excretion, both appeared in urine and were excreted at normal rates.

* * * * *

If one of the dyes, neutral red, is excreted by the tubular apparatus, while the other, phenol red, is not excreted in any significant amount by these elements of the kidney but only by the glomeruli, then destroying the function of the tubules should affect the rates of excretion in a very different manner.

Such an experiment involves the complex question of abnormal activity of the tubule epithelium and is one which we shall discuss and study more fully in studies on experimental nephritis in the frog to be published later. Höber (2) has done a considerable amount of work on this problem in his studies of the physiological activity of the kidney and we can refer to his findings in applying the method to our immediate problem.

Höber has shown that when the tubule cells are anesthetized with urethane, their characteristic activities cease. There is no longer an absorption of water from the lumen of the tubule, hence a diuresis results. Sugar, which Wearn and Richards (5) have shown to be present in the glomerular filtrate, is also not absorbed from the urine as it passes down the tubule lumen, and hence appears in the urine, and the absorption of NaCl is also decreased. The urine, therefore, as a result of the abolition of tubular absorption, approaches in its constitution the character of the perfusion fluid.

In our experiment the rate of excretion of the two dyes was determined when such conditions had been produced by the action of urethane. Fig. 5 shows the result of such an experiment. Neutral red was supplied in the first 2 periods to

the glomeruli alone and, as in the previous experiments, was excreted through them at a very low rate, namely, .02 mg. per hour. The dye was then led to the tubular system and in three periods (3, 4, 5) an hourly rate of excretion 30 times as high as previously, or .60 mg. per hour, was attained. The urine during all these periods was normal in every way; the amount was not excessive, sugar was not present and its salt concentration was below that of the perfusion fluid. Urethane was

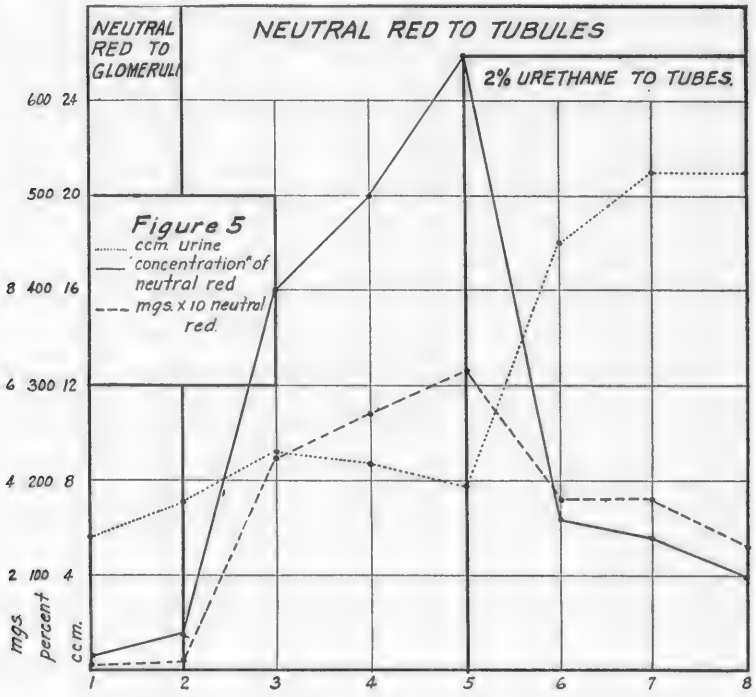

FIG. 5

now added in 2 per cent concentration to the perfusion fluid which was passing to the tubules. There was an immediate drop in the rate of excretion of the neutral red to about one-half its former figure (Period 6) and the rate continued to decrease during the next 2 periods, to a final figure of .22 mg. per hour, or one-third the value found under normal optimum conditions. All the evidence of tubular damage as described by Höber were present; there was a marked diuresis, sugar appeared in the urine and the salt concentration approached that of the perfusion fluid.

A similar experiment was now performed with phenol red. The results are shown in Fig. 6. The phenol red was introduced at once in this case into the glomerular circulation and as before was excreted at the relatively high rate of .3

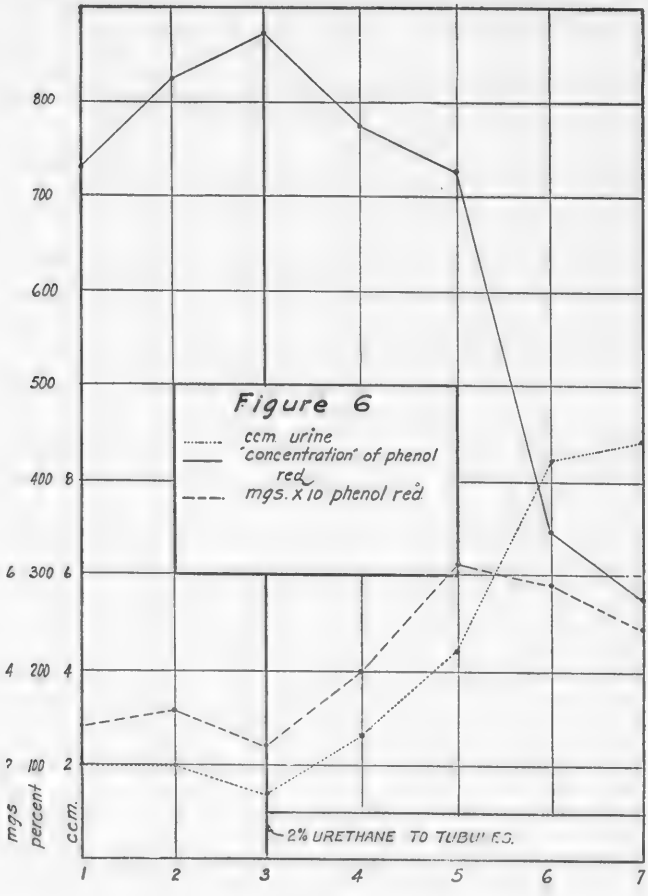

FIG. 6

mg. per hour. The urine contained no sugar, the salt concentration was only 40 per cent of that of the perfusion fluid and the amount of water was moderate. The same concentration of urethane, 2 per cent, was then introduced into the fluid which passed to the tubules. As the chart shows, in Period 6, sugar appeared in

the urine, the rate of water excretion increased 4 fold and the salt concentration rose, all evidences of tubular damage. The rate of excretion of phenol red, however, not only remained undepressed but actually increased.[2] The result is therefore the opposite of that obtained when neutral red was excreted, and gives further evidence which indicates the antithesis in the condition of excretion of the two dyes.

DISCUSSION

Since Nussbaum's original description of the double nature of the blood supply to the amphibian kidney and his experiments, based on this finding concerning the function of the tubules and glomeruli, numerous criticisms have appeared of the basic contention that the blood supply of the two systems is independent. One of the most recent of these is that of Smith (7) who from the result of certain experimental procedures comes to the conclusion that such rich anastomoses occur between the two systems that any interference with either the venous or the arterial systems will cause the unobstructed blood stream to supply both capillary systems. Perfusion experiments as done by many investigators, in so far as the renal portal vein is concerned do not therefore perfuse the kidney in the sense of supplying the tubule through a capillary bed.

A great deal of discussion would be necessary if the details of these experiments of Smith were to be adequately compared with experiments of other authors which are held by them to show the contrary results. For our purpose this can be avoided, for the good reason that our experiments are so designed and their results are of such a nature that in themselves they answer the objections of Smith and others. There is no doubt from our results that the excretion of the dyes is strikingly different when led to the kidney by the arterial and venous routes respectively, and that these differences are the converse of each other with the two dyes. In perfusion of phenol red through the renal portal system for example, the dye did not appear in significant amounts in the urine. This lack of excretion could not be, as Smith's objections might lead one to suppose, because it was passing through

[2] Hayman and Richards (6) have shown that the tubules absorb most dyes, including phenol red, so that abolition of the absorptive function of renal epithelium would increase the amount excreted under such conditions. A more detailed discussion of this point will be given in our studies on experimental nephritis.

the kidney directly to the post caval vein without passing through any capillary bed in the kidney, *because identical perfusion with neutral red through the same system was followed by the excretion of large amounts of dye.* And one can hardly suppose that this excretion of neutral red was the result of fluid reaching the glomeruli by anastomoses from the vein, for the direct introduction of the neutral red into the glomeruli was followed by the excretion of only a small amount.

Since Nussbaum's time there has been no question that anastomosis exists between the two systems. As Höber's experiments have shown (2) leakage from the glomerular system to the tubular is greater than the reverse flow from the venous capillaries to the glomeruli *under normal condition of relative pressures.* With abnormal relations of pressure in vein and artery this leakage of fluid and dye is increased, as our experiments with carbon particles and those of Smith and many others show. Furthermore, in the case of phenol red the apparent excretion of the dye, by the tubules, increases as this leakage increases. It may be true that this output of dye is due to the actual excretion of a small amount through the tubular system into which it is first introduced and that the dye is therefore excreted by both glomeruli and tubules as Tamura and his coworkers (8) have concluded from another type of experiment. But the increase in this apparent tubular excretion coexists with a demonstrable increase of leakage to the glomeruli, through which phenol red is readily excreted, and this fact leads us to believe that the excretion by the tubules is apparent rather than real. The converse we believe to be true of neutral red, the leakage which produces the apparent glomerular excretion of this dye being from the glomeruli to the tubules through the cells of which, as experiment shows, it is readily excreted.

In conclusion we wish to emphasize an important difference in the experiments described here from the majority of the previously reported perfusions of the frog kidney. The high arterial pressure as compared to that used by most investigators, combined with the size of our frogs, often 1000 grams in weight, has allowed us to obtain much larger samples of urine than those usually reported in such experiments. Their volume is, however, not abnormal, as Adolph (9) has shown that the average normal rate of water excretion by the frog when there is an excess of fluid available is 1.3 per cent of their weight per hour. This would mean that with our frogs a perfusion

which is normal in its production of water should produce from 7 to 13 cubic centimeters of urine per hour. No objection can be raised to the pressure in the glomerular circulation, which is high as compared to that which exists in the living frog, since it produces no abnormality in the urine. Repeated tests showed that the glomerular membrane was still intact, retaining gum arabic or albumin which had been added to the perfusion fluid, nor did the semi-colloidal neutral red pass through under this pressure, as our experiments have shown. All the other constituents of urine appeared in normal concentration and amount. Under the conditions of our experiments, therefore, the kidneys functioned in a normal manner, a statement which can not be made of experiments where volumes of urine of .19 cubic centimeter were excreted per hour. The added difficulty of accurate quantitative measurements under such conditions is also obvious.

CONCLUSIONS

1. Phenol red and neutral red are excreted by the perfused frog's kidney by different routes.

2. Phenol red is excreted chiefly through the glomerulus, neutral red through the tubules.

3. Some slight excretion of each of these dyes by the converse mechanism is possible, though there is no evidence in our experiments that necessitates such a conclusion.

4. The importance of methods leading to the production of a normal volume of urine by the perfused kidney is emphasized.

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FURTHER NOTES ON THE FILTRATION OF THE VIRUS OF VACCINIA

By HUGH K. WARD

(*From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston*)

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In a previous note,[1] attention was drawn to a phenomenon of filtration—first noticed by Bronfenbrenner[2] in connection with bacteriophage—*viz.*, that if tissue containing the viruses of either herpetic encephalitis or vaccinia are suspended in broth instead of saline, filtration of the viruses through diatomaceous filters is greatly facilitated, active filtrates being constantly obtained.

No further work has been carried out with the virus of herpetic encephalitis except to ascertain the minimal lethal dose for a rabbit of the Berkfeld V filtrate. If a 5 per cent emulsion is made in broth of the brain of a rabbit dying of herpetic encephalitis, and the emulsion centrifuged, the minimal lethal dose of the supernatant fluid may be about 0.0008 cc., when injected intracerebrally, whereas the minimal lethal dose of the Berkfeld V filtrate of a supernatant fluid as virulent as this is about 0.06 cc. Thus only about 1 per cent of the virus passes through the filter.

Many filtrations of vaccinia pulp have been carried out since the previous communication, and a very active filtrate was obtained from each lot of material that has been tested, if certain conditions were observed.

Testing of the Filtrate

Every filtrate has been tested carefully for sterility (*i.e.*, absence of visible bacteria), by taking at least 1 cc. of filtrate, inoculating it into hormone broth and observing it during many days of incubation. Since the pulp, as taken from the calf, contains many and various organisms, it was not thought necessary to add

[1] Ward, H. K., and Tang, F.-F., *J. Exp. Med.*, 1929, **49**, 1.

[2] Bronfenbrenner, J., *J. Exp. Med.*, 1927, **45**, 873.

any test organisms to the material to be filtered. No growth was ever observed in the filtrate growth-control tube in the course of these experiments.

Each filtrate was titrated on the skin of rabbits, and a measure of its activity was thus obtained. Titrations of the same filtrate on different rabbits gave nearly identical results, so that it was felt that a comparison of the titers of different filtrates could be justly made, even if they were not all carried out on the same animals. In carrying out the titrations, the filtrates were diluted 1:4, 1:16, 1:64, 1:256, etc., and 0.2 cc. of the undiluted filtrate and of the successive dilutions were injected intradermally into the shaven skin of normal, well-grown rabbits. Six titrations can easily be carried out on the same rabbit, with three rows of injections on either side of the vertebral column. A No. 27 gauge needle is used, and care taken that the injection is made actually into the skin, and not into the subcutaneous tissue. The reactions are read on the fifth or sixth day after injection. The maximum reaction is an inflamed swelling of firm consistency, about 12 mm. in diameter, sometimes necrotic in the center. As one approaches the end-point of the activity of the filtrate, the reactions become smaller, the smallest detectable reaction being a pin-point papule. The reactions caused by the filtrate are indistinguishable from those following the injection of the unfiltered emulsion of vaccine pulp.

The intradermal was used in preference to the scratch method in order to obtain more accurate readings.

The Conditions Necessary for Obtaining a Filtrate of Maximum Activity

The technique in use at present will first be described, and then the various factors will be discussed.

Present Technique.—The scrapings from the calf—technically called the pulp—are emulsified and filtered as soon as possible after their removal from the animal. The pulp is weighed roughly, placed in a glass mortar and thoroughly ground with fragments of Pyrex capillary tubing. Enough hormone broth is then added to make a 5 per cent emulsion of the pulp. The broth is added slowly at first, and then in bulk, grinding being continued the whole time in order to obtain as homogeneous an emulsion as possible. The emulsion is then centrifuged at about 2,000 r.p.m. for 30 minutes, and the supernatant poured off. The supernatant fluid is then filtered through a Berkefeld V filter at a negative pressure of about 30 cm. of mercury. The filtrate is pipetted off into tubes and kept in the refrigerator. A small quantity of the filtrate is incubated with hormone broth for some days to test for the presence of bacteria.

The reactions following the intradermal injection into a rabbit of 0.2 cc. of this filtrate and of its successive dilutions, should be as given in Table I.

The Pulp

It was a matter of considerable surprise to find that, if the pulp was kept in the refrigerator for a few days before filtration, the filtrate was barely active, although the unfiltered emulsion had lost none of its activity. Further, the filtrate was inactive, if the pulp was ground up at once and glycerine added before storing in the refrigerator for a few days, and then filtering. Whatever the details of the mechanism of this phenomenon, it was thought likely that the change in filterability was bound up with oxidation of the tissue. And that this appears to be the case is shown by the following experiment:

Fresh pulp was ground up, emulsified in hormone broth and the emulsion centrifuged. The supernatant fluid was then poured off and divided into two parts.

TABLE I

Dilution of filtrate	Reaction	Dilution of filtrate	Reaction
1/1	++++*	1/1,024	+++
1/4	++++	1/4,000	++
1/16	++++	1/16,000	+
1/64	++++	1/64,000	±
1/256	++++	1/256,000	0

* Here and in the following tables, ++++ denotes the maximum reaction, and ± a pin-point papule.

One part was kept in an open tube in the refrigerator; to the other part cystein was added so that a final concentration of 1:1,000 cystein was present, the tube was sealed with vaseline and placed in the refrigerator. This concentration of cystein is ample to prevent oxidation in a closed tube, methylene blue being completely reduced. Both tubes were kept in the refrigerator for 7 days and then filtered through Berkefeld V filters. Both unfiltered emulsions and both filtrates were then titrated. The unfiltered emulsion in the open tube, the unfiltered emulsion in the closed reduced tube, and the filtrate of the latter all proved to be active up to the usual dilution, but the filtrate of the emulsion in the open tube was only very feebly active, giving rise to but pin-point papules in the strongest dilutions. Whether this alteration in filterability in the oxidised tube was due to a lowering of the hydrogen ion concentration and consequent change in the charge on the virus has not yet been ascertained.

The brain of a rabbit infected with neuro-vaccine gave a filtrate of approximately the same activity as the filtrate of calf epidermal lesions.

The Filter.—The filters used in these experiments were the 2½ inch size Berkefeld V filters, autoclaved before using. The majority of them were new filters, but some had been used two or three times. The negative pressure, under which the filtrations were carried out, varied, but usually was about 30 cm. of mercury, although occasionally it was as high as 60 cm., if filtration was slow. The rate of filtration varied with different filters, for reasons which are not understood, but unless filtration was very slow, the rate did not affect the activity of the filtrate. The rate of filtration of course gradually slows, and no more than 50 cc. were ever passed through one filter. Some of the broth filtrates show a slight opalescent haze, but this apparently has no relation to the activity of the filtrate.

TABLE II

Dilution of filtrate	Sand filtrate	Carborundum filtrate	Pyrex glass filtrate
1/1	++++	++++	++++
1/4	++++	++++	++++
1/16	++++	+++	++++
1/64	+++	++	++++
1/256	++	+	++++
1/1,024	+	±	+++
1/4,000	0	0	++
1/16,000	0	0	+
1/64,000	0	0	±

In one experiment a Berkefeld N filter and a Chamberland L1. bis candle were tried. In the case of the Berkefeld N, the undiluted filtrate gave rise to a slight reaction; in the case of the Chamberland, the filtrate was entirely inactive.

The Grinding Material.—In the preliminary experiment sand was used in the glass mortar to help in the grinding of the pulp. Carborundum was tried in place of the sand, but was no improvement. With the idea that perhaps these substances had an absorptive action on the virus, ground up glass was tried. Whatever the reason may be, it was found that Pyrex glass fragments were a definite improvement over sand or carborundum as a grinding material. This is shown in Table II.

To prepare the glass fragments, small pieces of 7 mm. Pyrex tubing are drawn out into short capillaries, which are then cut into 25 mm. lengths and dry sterilized. Before placing the pulp in the mortar, the capillary tubing is ground up roughly, so that, when the pulp is added and grinding commenced, flying fragments of glass do not scatter the pulp over the bench.

The Broth.—The hormone broth is prepared in a somewhat different manner to that originally described by Huntoon.[3] 400 gm. of minced beef heart, 10 gm. of peptone and 5 gm. of salt are boiled for 15 minutes in 1 liter of water. The meat is then removed by straining through a wire sieve, 24 cc. of N/1 NaOH added, and the broth autoclaved at 15 lbs. for 30 minutes. The broth is then allowed to stand over night. Next day the supernatant fluid is syphoned off, adjusted to pH 7.6 and autoclaved at 15 lbs. for 15 minutes.

Meat infusion broth without peptone, a buffered peptone solution, and saline have all been tried, but none of these are as effective a menstruum in which to suspend the pulp as hormone broth. This is shown in Table III.

TABLE III

Dilution of filtrate	Hormone broth filtrate	Infusion broth filtrate	Peptone filtrate	Saline filtrate
1/1	++++	++++	++++	++++
1/4	++++	++++	++++	++++
1/16	++++	+++	++	++++
1/64	++++	++	+	++
1/256	++++	+	0	+
1/1,024	+++	0	0	0
1/4,000	++	0	0	0
1/16,000	+	0	0	0
1/64,000	0	0	0	0

No further analysis than this has been made of the substance in hormone broth which aids filtration of the virus, but this subject is being investigated by a colleague in the laboratory.

The Properties of the Virus-containing Filtrate

Activity.—As one of the underlying ideas in this work was to obtain a sterile filtrate for use in human vaccination, it was of importance to know in the first place how its activity compares with that of the unfiltered, non-sterile vaccine in general use. Table IV shows this comparison, which was carried out on the same rabbit on adjacent areas of skin. This table shows that the unfiltered virus in general

[3] Huntoon, F. M., *J. Infect. Dis.*, 1918, **23**, 169.

use for vaccination is about sixteen times more active than the virus filtrate prepared according to the technique described above.

Survival.—In the second place, if the filtrate is to be used as a vaccine, it must be able to survive for a reasonable length of time in the refrigerator. Accordingly, tests were carried out from time to time to see if it was possible to detect any deterioration of specimens kept in the ordinary electric refrigerator. A filtrate prepared and titrated

TABLE IV

Dilution	Vaccinia filtrate	Unfiltered vaccine
1/1	++++	++++
1/4	++++	++++
1/16	++++	++++
1/64	++++	++++
1/256	++++	++++
1/1,024	+++	++++
1/4,000	+++	++++
1/16,000	++	++++
1/64,000	±	+++
1/250,000	0	++
1/1,000,000	0	±

TABLE V

Dilution of filtrate	Filtrate titrated Nov. 6	Filtrate titrated Jan. 22
1/1	++++	++++
1/4	++++	++++
1/16	++++	++++
1/64	+++	++
1/256	++	+
1/1,024	±	0

on Nov. 6 was kept in the refrigerator (about 8°C.) and titrated again on Jan. 22. In this period of 11 weeks, it had deteriorated to about half of its original strength, as is shown in Table V.

Another tube of the same filtrate, kept in contact with the ice-block, but not quite frozen, had hardly deteriorated at all in this length of time, so it is evident that if the filtrate is frozen, it can be kept for long periods of time without any appreciable loss of its activity, and that it only deteriorates slowly at temperatures just above freezing.

As the filtrate loses its activity so slowly in the refrigerator, the influence of glycerine was investigated by keeping the tubes at room temperature. This experiment brought out the fact that in a mixture of equal parts of glycerine and vaccinia filtrate, the virus died out sooner than in control tubes containing vaccinia filtrate alone.

The Nature of the Virus.—Since the virus was suspended in a fluid free from tissue cells and débris, an attempt was made to determine whether it was possible to concentrate the virus by centrifugation.

10 cc. of the filtrate was placed in a conical-ended centrifuge tube and centrifuged at about 2,500 r.p.m. for 4 hours. The supernatant fluid was pipetted off, leaving about 0.1 cc. in the point of the tube. Both the supernatant and the residuary 0.1 cc. were then titrated out. The result is given in Table VI.

TABLE VI

Dilution	Supernatant fluid	Residuary fluid
1/1	++++	++++
1/4	++	++++
1/16	+	++++
1/64	0	++++
1/256	0	+++
1/1,024	0	+

Centrifugation thus causes a definite concentration of the virus at the bottom of the centrifuge tube. A more striking result was obtained with a filtrate which showed a slight haze when examined before centrifugation, and a distinct deposit in the point of the tube after centrifugation, but this result may have been due to the carrying down of the virus by the deposit. Certainly neither the haze nor the deposit was virus alone.

Microscopic examination of neither the whole filtrate, the residuary fluid nor the deposit yielded anything that could be said definitely to be the virus, although suggestive forms were seen in Giemsa preparations and in the dark field.

Complement Fixation.—A careful complement-fixation test was carried out by Mr. Seastone, using a centrifuged concentrate of the vaccinia filtrate as antigen, and an immune calf serum which had a

strong neutralizing action on the filtrate as antibody. The experiment was completely negative.

DISCUSSION

These experiments have demonstrated once again the ease with which a very active sterile vaccinia filtrate can be obtained from vaccinia pulp, if certain conditions are observed. The essential factors in the preparation of such an active filtrate are

1. The use of fresh vaccinia pulp.
2. The grinding of the pulp with Pyrex glass fragments in a glass mortar.
3. The emulsification of this ground-up pulp in hormone broth.
4. Centrifugation of the emulsion and the passage of the supernatant fluid through a Berkefeld V filter.

In the filtration of a virus, assuming that the virus is particulate in nature, there are obviously three fundamental factors governing the passage of the virus through the pores of the filter—the size of the virus; the size of the pores of the filter; and what might be termed its "freeness," meaning by that term its ability to pass through the pores of the filter with no other impediment than that due to its size. Its passage could be prevented either by its adherence to, or retention in larger particles in the fluid to be filtered, or its adherence to the walls of the filter pores. It is probable that hormone broth is more efficient than saline in preventing the adherence of the virus to the walls of the pores, although the actual mechanism of its action is unknown. The glass fragments are probably more efficient than sand in grinding up the virus-containing tissue by reason of their sharp edges, but another reason may be that the virus adheres to the sand, and not to the glass. Another phenomenon which needs further investigation is the action of oxidation in preventing filtration of the virus from an emulsion of the pulp which has been exposed to air for some time, although here again it is probable that a change in the charge on the virus makes it adhere to the pores of the filter.

Although not as active as the unfiltered non-sterile emulsion which is in general use for vaccination against small-pox, a sterile filtrate can be prepared which approaches the former in strength and is probably quite active enough for the purposes of immunization. When

inoculated on to the skin of a calf by the scratch method, the resulting lesions are indistinguishable from those from standard vaccine. A suitable technique for human vaccination has yet to be worked out. Theoretically, there should be more certainty and no danger with intradermal inoculation, as the fluid to be injected contains nothing living but the virus. If the scratch method be used, it would doubtless be necessary to add some substance such as glycerine to make the fluid sticky, as the filtrate alone (consisting mainly of broth) runs off the skin too readily.

Recent work has thrown considerable doubt on the presence in serums which neutralize filtrable viruses *in vivo* of antibodies reacting *in vitro*, and certainly no evidence was obtained in these experiments for the presence of agglutinins, precipitins, or complement-fixing antibodies in a serum which neutralized *in vivo* a filtrate containing the vaccinia virus, using the filtrate as the antigen in the *in vitro* experiments.

Previous differential centrifugation experiments with vaccinia virus have always shown a concentration of the virus in the lower part of the centrifuge tube, but as they have been carried out with an unfiltered emulsion, it could be argued that the virus was simply carried down or contained in the cells and cellular detritus. However, the experiment outlined above—in which a clear filtrate was centrifuged at only a moderate revolution rate—showed a fairly definite concentration of the virus in the lowermost layer. This and the almost complete retention of the virus by a Berkefeld N filter point to it as within the limits of visibility under the microscope. Suggestive forms were seen, but proof of their identity with the virus is still to seek.

It has also been shown in these experiments that at least one filtrable virus can survive perfectly well without the addition of glycerine, if it has been freed from its containing tissue by filtration, and indeed the addition of glycerine seems to be somewhat detrimental to its survival under these conditions.

CONCLUSIONS

1. A very active filtrate can be obtained from vaccinia lesions by grinding up the fresh tissue with glass fragments, emulsifying in hor-

mone broth, centrifuging the emulsion and filtering the supernatant fluid through a Berkefeld V filter.

2. The sterile filtrate so obtained has been shown by comparative titration on rabbits to have about one-sixteenth of the activity of the non-sterile emulsion used in human vaccination.

3. Centrifugation of such a filtrate shows a partial concentration of the virus in the lowermost layer.

4. The virus survives for a long time, if the filtrate is kept near the freezing point, and probably will survive indefinitely if kept frozen. The addition of glycerine is not necessary.

The author wishes to express his grateful thanks to Dr. B. White and Dr. A. L. Reagh of the Massachusetts Antitoxin Laboratory for the readiness with which they placed vaccine pulp at his disposal on numerous occasions.

ATTEMPTS TO REPRODUCE RHEUMATIC FEVER IN ANIMALS

By LOUIS GROSS, M.D., LEO LOEWE, M.D., and BENJAMIN ELIASOPH, M.D.

(*From the Laboratories of Mount Sinai Hospital, New York*)

Plates 1 to 3

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INTRODUCTION

With the assumption that rheumatic fever* is due to some infectious agent, the isolation and identification of the agent has been attempted by bacteriological and immunological methods as well as by animal experimentation. Of the various organisms which have been described in connection with this disease, the streptococcus has held foremost attention and is at present the only one which deserves serious discussion with respect to etiology. An analysis of the present status of our knowledge on the cause of rheumatic disease, with few and practically negligible exceptions, amounts to an evaluation of the possible rôle played by the streptococcus.

The association of the streptococcus with rheumatic disease was first brought into prominence by Poynton and Paine.[1] Whilst many articles have been published on the subject, up to recent years those investigators who had conducted the greater number of animal experiments with streptococci paid little attention to the biochemical characteristics of the organisms with which they were dealing. It is particularly for this reason that it is difficult to analyze and evaluate much of the past literature on this subject.

The first obstacle which presents itself in the acceptance of the streptococcus as the etiologic agent of this disease is that, whereas many investigators claim an extraordinarily high percentage of positive blood cultures (Herry,[2] Freund and

*We feel that the terms "Acute rheumatic fever, Acute articular rheumatism, Rheumatic pancarditis," etc., are each in themselves too limited to cover descriptively all the possible manifestations of this disease. It would seem that the term "Rheumatic Disease" would be preferable inasmuch as this condition undoubtedly exists during afebrile periods.

Berger,[3] Clawson,[4] Suranyi and Forro[36]), many equally careful investigators have obtained a strikingly small percentage of positive blood cultures (McCrae,[5] Philipp,[6] Cole,[7] Harrison,[8] Swift and Kinsella[9]). These discrepancies, however, may be explained possibly on the basis of different technic employed.

The next objection lies in the variety of streptococci which have been found. Thus, while many investigators have obtained from various sources (though principally blood cultures) the *Streptococcus viridans* (Swift and Kinsella,[9] Freund and Berger,[3] Clawson[4]), some have obtained a non-methemoglobin producing organism (Small,[10] Birkhaug[11]) and some have obtained an occasional hemolytic streptococcus (Clawson,[4] Rosenow[12]). The difficulty is further enhanced by the fact that in the hands of most observers the organisms do not appear to fall into one serological group. The outstanding exceptions to this are: Clawson,[4] who found 7 of his 10 *Streptococcus viridans* strains to fall into one homogeneous serological group, Small,[10] who found that 31 strains of "*Streptococcus cardioarthritidis*" obtained from blood cultures, tonsils, feces, etc., fall into one serological group, and Kreidler,[13] who found that 107 strains of "*Streptococcus cardioarthritidis*" obtained from similar sources also fall into one serological group. It is noteworthy that none of these investigators mention absorption tests to prove that they were not dealing with group agglutination.

The only explanations which would still be consistent with such diametrically opposed findings as regards the streptococcus would be to assume either with Rosenow[12] that the streptococcus need not be of a specific type but with specific affinities, or that in this disease the human tissues respond by a specific reaction to non-type-specific agents, particularly under certain hypersensitive states. The latter hypothesis has been voiced by a number of workers and has recently been stressed by Swift[9] and his coworkers.

Another more outstanding discrepancy lies in the fact that, whereas many have claimed the reproduction by means of streptococci, etc., of one or more of the following lesions: endocarditis, arthritis, chorea, and, more significantly, Aschoff-like bodies (Wassermann, Westphal and Malkoff,[14] Meyer,[15] Poynton and Paine,[1] Shaw,[16] Beattie,[17] Rosenow,[12] Coombs, Miller and Kettle,[18] Jackson,[19] Harzell and Henrici,[20] Henrici,[21] Small,[10] Belk and Jodzis,[22] etc.), others have been able to reproduce similar lesions with a variety of organisms not from rheumatic sources (Cole,[7] Horder,[23] Davis,[24] Thalhimer and Rothschild,[25] Cecil,[26] Topley and Weir[27]).

In connection with the alleged production of Aschoff bodies, it must be pointed out that not only are the published photomicrographs of these lesions in animals entirely unconvincing, as has been pointed

out by some of the last named observers, but, also, that Miller[28] called particular attention to the spontaneous occurrence of inflammatory foci in the myocardium of the rabbit and of the guinea pig and suggested that these lesions may have been mistaken for Aschoff bodies in the past.

The interesting work by Birkhaug[11] which has recently been reported may be mentioned. Birkhaug has obtained a toxin from a non-methemoglobin forming streptococcus which he claims produces a positive skin reaction in a higher percentage of rheumatic patients than in non-rheumatic subjects. Injected into his own wrist joint (after previously sensitizing himself with subcutaneous injections of the toxin), it produced a wandering arthritis resembling rheumatic polyarthritis. No definite conclusions can, however, as yet be drawn from this work. Birkhaug's work on skin reactions has been confirmed by Kaiser,[29] Swift,[9] and his coworkers, and by Edith Irvine-Jones.[30]

Swift and his coworkers, however, showed that rheumatic patients are equally sensitive to filtrates from *Streptococcus viridans*, as well as to vaccines and nucleoproteins. In fact, they hold that rheumatic patients show an intensified reactivity (hyperergy) to these products and suggest the possibility that rheumatic disease may be the expression of some such "hyperergy" produced by a variety of streptococci.

Edith Irvine-Jones[30] found that filtrates made from streptococci of the alpha, beta and gamma variety,[37] whether obtained from throats of normal subjects or from those suffering of rheumatic disease, produced in the latter a higher percentage of positive skin reactions than in normal controls.

It is not our intention to review the literature on rheumatic disease in detail. It is obvious, however, from this brief survey that the subject is still in a chaotic state. For this reason, we thought it advisable to repeat some of the work reported in the past, particularly with the object of reproducing the disease in animals. Moreover, there was to be used a large number of animals of different species and a variety of materials obtained from rheumatic patients (see "experimental" below), including "pedigreed streptococci," *i.e.*, organisms obtained from patients in whom autopsy, biopsy (subcutaneous nodules), or clinical follow-up established the fact that they had suffered of rheumatic disease at the time that the cultures were made. In addition, there was to be employed a variety of procedures calculated to sensitize the animals or render them susceptible to the disease.

EXPERIMENTAL

The material and technique employed in these studies were as follows:

Inocula.—I. Twenty strains of streptococci whose source and cultural characteristics are shown in Table I. In order to indicate that these organisms were obtained from patients suffering of rheumatic fever, they will be referred to briefly as "Rheumatic streptococci." Many of the organisms were passed through animals in order to raise their virulence (see appendix). The procedure adopted was to place the organisms in pure culture with broth in collodion sacs. The sacs were transplanted into the peritoneal cavities in animals of the same species into which the organisms were ultimately to be injected. In the case of rabbits, the strains were passed through the knee joints of successive animals.

II. Apart from the streptococci, there was used other material from rheumatic cases, such as, whole blood, plasma and buffy layer, serum, pericardial and hydrocele fluid, and filtrates from tonsils, subcutaneous nodules, lymph nodes and nasopharyngeal washings. These were obtained from sources indicated in Table I. The lymph nodes, subcutaneous nodules, and tonsils were each ground with sterile sand, extracted with saline and filtered through Berkefeld candles. The filtrates were injected in the manner indicated in the protocols.

Animals.—Seven species of animals were employed, *viz.*, rabbits, guinea pigs, dogs, cats, swine, sheep and calves. Most of the rabbits employed and all of the dogs, cats, and guinea pigs were full grown animals. The sheep were approximately 8 months old. The swine and calves were used shortly after being weaned.

In the several groups of animals employed a variety of procedures were instituted in different combinations.

Portal of Inoculation.—Injections were made intravenously, intratracheally, intracardially, into the tonsils, joints, pericardium, peritoneal cavity and nasopharyngeal mucous membrane.

Physical State of Inoculum.—Washings of 18 hour agar slants, 18 hour cultures in various fluid mediums,* organisms agglutinated in homologous rabbit sera, organisms incubated (sensitized?) in sera from patients suffering of rheumatic disease, organisms mixed with finely divided agar, organisms suspended in 5% gum tragacanth.

Other Procedures.—Infected agar masses were injected subcutaneously into some animals. Large doses of Digitan were injected intravenously into some animals with the object of poisoning the heart muscle and thus lowering its resistance to infection.† In many animals an external jugular or a femoral vein was either

* Noguchi, Rosenow, dextrose veal infusion broth, dextrose mutton infusion broth, human hormone medium.

† These injections were carried out in rabbits only. Each rabbit received one intravenous injection of 0.5 cc. of Digitan per kilo twice a week to begin with. The dose and number of injections were increased gradually so that after three weeks the animals were receiving 1 cc. per kilo daily.

crushed or painted with 20% silver nitrate. The object of this was to produce a thrombus which might become the seat of infection with the organism subsequently injected intravenously. It was hoped in this way to produce a constant blood stream infection. Many animals were injected intraperitoneally with large doses of saturated Trypan blue in saline (20 cc. per kilo of rabbit injected weekly). The object of this procedure was to attempt a blockade of the reticulo-endothelial system in order to lower resistance to infection. Daily temperature readings were taken but only abnormal temperatures are indicated in the protocols.

For convenience of analysis the experiments will be summarized in three groups, *viz.*, Group A, Group B, Group C. In each group, animals of different species were used with the object of finding a species susceptible to the organism or virus and because it was hoped that the existence of blood vessels in the valves of some of these species (calves, sheep, swine) in fairly high per cent might furnish a means of localizing an infective agent in the valves.

In some of the groups the experimental procedures were relatively simple, hence an abbreviated summary of the technic will be given for these groups. Where the procedures were more complicated, detailed protocols will be given in the appendix.

Group A

Dietrich[31] has claimed that after sensitizing rabbits by means of successive intraperitoneal injections of first, dead organisms (staphylococci) then live organisms, followed after a suitable interval of time by a small intravenous injection of live organisms, he was able to obtain a high per cent of verrucous endocarditis in rabbits. We attempted to repeat this method of sensitization in a variety of species, using streptococci obtained from patients suffering of rheumatic disease, in the hope of producing verrucous endocarditis with, possibly, other rheumatic clinical and pathological manifestations.

Twenty-four rabbits, 12 guinea pigs, 5 dogs, 9 cats, 2 swine, 1 sheep and 2 calves were employed in this experiment. These were divided in three equal subgroups. The first subgroup was injected with Strains 7A-MB and 26A-IS. The second subgroup was injected with Strains 5A-MK, 9A-IS, 3A-EB, 6A-MT, 10A-IS, 4A-RD, and 11A-RG. The third group received Strain 8A-NG. Since, however, there were no conspicuous differences in the results found in these subgroups the procedures detailed in the following pages will refer only to "rheumatic streptococci" without indicating the identity of the individual strains.

TABLE I

No.	Nature of material	Age	Joints	Pericarditis	Nodules	E.C.G.	X-ray	Attacks	Clinical diagnosis
1A-RG	Pos. bld. clt. *Strep. gamma**	10			4+	Neg.	Pos.††	1	Rheum. heart dis.
2A-JW	Pos. bld. clt. *Strep. mitis*†	7				Neg.	Pos.	2	Rheum. heart dis. Chorea
3A-EB	Pos. bld. clt. *Strep. saliv.*	6		+			Pos.	1	Acute rheum. dis. Pericard.
4A-RD	Pos. bld. clt. *Strep. fecal.*	9				?	Pos.	1	Acute rheum. dis.
5A-MK	Pos. bld. clt. *Strep. fecal.*	13	R. elbow, foot				Pos.	2	Acute rheum. heart dis.
6A-MT	Pos. bld. clt. *Strep. gamma*	5				?	Neg.	1	Acute rheum. dis.
7A-MB	Pos. bld. clt. *Strep. fecal.*	7	L. hip	+	+			1	Acute rheum. dis.‡°
8A-NG	Pos. bld. clt. *Strep. fecal.*	9	R. knee, foot	+		Pos.**	Pos.	2	Acute rheum. dis. Pericard.
9A-IS	Pos. bld. clt. *Strep. fecal.*	11		+		Neg.	Pos.	4	Acute rheum. dis. Chorea
10A-IS	Pos. bld. clt. *Strep. fecal.*	28	Hips, knees and ankles			?	Pos.	1	Acute rheum. dis.
10B-IS	Buffy layer of cit. bld.								
11A-RG	Pos. bld. clt. *Strep. fecal.*	28	Mult. arthrit.			Neg.	Pos.	2	Acute rheum. dis.
12A-LH	Pos. bld. clt. *Strep. gamma*	45				Pos.		6	Acute rheum. dis. Aur. Fib.
13A-R to 17A-R, Pos. bld. clts., Swift, *Strep. virid.* = his B-39, 38-D, B-38, A-141, A-135, respectively.									
18A-VF	Sterile peric. fld. P. M.	27		+		Pos.		2	Acute rheum. dis. Pericard.
18B-VF	Cit. and defib. bld.								
19A-RB	Cit. and defib. bld.	12		+		Pos.		3	Acute rheum. dis. Aur. Fib.
20A-SY	Tonsil filtrate	33				Pos.	Pos.	3	Rheum. dis.
21A-VS	Sterile bld.	9	Mult. arthrit.			Neg.	Neg.	1	Polyarthritis
21B-VS	Tonsil filtrate								
22A-YL	Sterile bld. clt.	19				L.V.P.	Pos.	3	Chr. cardio-valv. dis.
23A-MB	Sterile bld. clt.	32	Mult. arthrit.			L.V.P.		1	Polyarthritis
24A-AW	Sterile bld. clt.	12	Knees, feet			Neg.	Neg.	1	Acute rheum. dis.
25A-RG	Sterile bld. clt.	17	Ankles, wrists	+		?	Pos.	1	Acute rheum. Pericard.

26A-IS	Valve clt. *Strep. fecal.*	41				Pos.		2	Chr. cardio-valv. dis.‡
27A-SD	Valve clt. *Strep. ignav.*	16		+	+	Pos.	Pos.	2	Acute rheum. dis. Pericard.‡°
28A-FB	Tonsil clt. *Strep. mitis*	10		+	4+	Neg.	Pos.	3	Acute rheum. dis. Pericard.
29A-MM	Sterile peric. fld.	10		+	3+	?	Pos.	1	Acute rheum. dis. Pericard.‡°
29B-MM	Filt. of lymph node								
30A-MG	Hydrocele fld.	15		+		Pos.	Pos.	2	Acute rheum. dis. Aur. Fib.
31A-AW	Buffy layer of cit. bld.	5		+	+		Pos.	1	Chr. valv. dis. Ac. Pericard.
31B-AW	Tonsil filtrate								
31C-AW	Serum								
32A-AK	Cit. and defib. bld.	20				Pos.		1	Acute rheum. dis. Pericard.
32B-AK	Serum								
33A-HH	Sterile defib. bld.	35	Shoulders			Pos.	Pos.	6	Chr. cardio-valv. dis. Aur. Fib.
34A-JR	Tonsil filtrate	8						4	Chr. cardio-valv. dis.
35A-MS	Tonsil filtrate	11				Pos.	Neg.	4	Chr. cardio-valv. dis.
36A-HM	Serum	10			+	Pos.	Pos.	3	Ac. rheum. dis. Chr. valv. dis.
37A-JR	Filt. nasophar. wash.	37	Wrists and			L.V.P.	Neg.	1	Subacute rheum. arthritis
37B-JR	Cit. and defib. bld.		L. shoulder						
38A-MP	Tonsil filtrate	35	Shoulder				Neg.	1	Subacute rheum. arthritis
39A-EZ	Buffy layer of cit. bld.	15		+		Pos.	Neg.	3	Chr. cardio-valv. dis.
40A-AL	Filt. of nodule	12		+	+	?		2	Acute rheum. dis.‡°
40B-AL	Filt. of lymph node								

*All organisms in this table except 13A-R to 17A-R belong in the gamma group of Brown.[37] **Pos. E.C.G. = finding of either prolonged conduction time, A-V block, S-A block, or auricular fib. †Holman classification.[38] ††Pos. X-ray = mitral or aortic configuration, pericardial effusion, singly or in combination. ‡Confirmed by autopsy. °Aschoff bodies present.

Rabbits

Twenty-four rabbits received from 1 to 6 injections of "rheumatic streptococci." The first intraperitoneal injection consisted of an amount of saline suspension of killed* organisms equivalent to 3 sq. cm. of an 18 hour growth on glucose agar. In the following three intraperitoneal injections, given at 4 day intervals, the number of organisms was doubled each time. The fifth intraperitoneal injection was similar to the first except that living organisms were employed. The sixth injection was given intravenously after a 12 day interval and consisted of one loopful of an 18 hour agar slant of the organisms.

Only six of these rabbits lived long enough to receive an intravenous loopful. These rabbits were exsanguinated under ether anesthesia in from 2 days to 1 month after the last injection. One of the rabbits showed, microscopically, several small scattered areas of accumulations of lymphocytes and mononuclear cells in the heart muscle. Otherwise the gross and microscopic findings in all six rabbits were negative. The remaining 18 rabbits died in from 6 days to 4 weeks after the initial injection. Eleven of the rabbits gave completely negative results. Of the remaining seven, five died with purulent pleurisy and lobular or lobar pneumonia due to organisms other than the streptococci injected. Four showed, microscopically, small scattered areas of scarring in the heart muscle, two showed a low grade pericarditis with mononuclear cells. One showed small focal interstitial collections of lymphocytes in the myocardium. One showed an acute focal interstitial myocarditis. One showed a large, irregular, thrombotic mass on the posterior cusp of the mitral valve. This thrombotic mass contained no demonstrable bacteria. There were no evidences grossly or microscopically of embolic phenomena in any of the organs. It is rather interesting to note that this rabbit received only two intraperitoneal injections of dead, pooled organisms.

Guinea Pigs

Twelve guinea pigs received from 8 to 10 injections of "rheumatic streptococci." The first intraperitoneal injection consisted of an amount of a saline suspension of killed organisms equivalent to 1.5 sq. cm. of an 18 hour growth on glucose agar. In the following three intraperitoneal injections, given at 4 day intervals, the number of organisms was doubled each time. The next three intraperitoneal injections were similar to the first three injections, respectively, except that live organisms were employed. After a lapse of 1 week, the equivalent of one-half a loopful of an agar slant of live organisms was injected intravenously. Six of the guinea pigs received two more intravenous injections of live cultures each equivalent to 500 loopfuls of agar slant, at weekly intervals.

Two guinea pigs died before receiving the intravenous injection. The rest were bled out under anesthesia at intervals varying from 1 day to 4 months after

* Killed by weak phenol solution.

receiving the last injection. None of the animals presented clinical or gross pathological abnormalities. Microscopically, six showed focal interstitial accumulations of lymphocytes and large mononuclear cells in the heart muscle. There was a tendency toward perivascular localization of these inflammatory foci. They were also localized, apparently with predilection, in the auricular muscle at the base of the mitral valve.

Dogs

Five dogs received from 6 to 9 injections of "rheumatic streptococci." The first intraperitoneal injection consisted of an amount of a saline suspension of killed organisms, equivalent to 9 sq. cm. of an 18 hour growth on glucose agar. In the following three intraperitoneal injections given at 4 day intervals, the number of organisms was doubled each time. The next four intraperitoneal injections were similar to the first four injections, respectively except that live organisms were employed. After a lapse of 2 weeks the equivalent of three loopfuls (in 2 dogs, 300 loopfuls) of an agar slant of live organisms was injected intravenously.

The dogs were bled out under anesthesia in intervals of from 2 weeks to 4 months. One dog died of lobar pneumonia before receiving the intravenous injection of live organisms. The other dogs showed no gross or microscopic pathological lesions. Clinically, the dogs had been normal.

Cats

Nine cats received each from 8 to 9 injections of "rheumatic streptococci." The first intraperitoneal injection consisted of an amount of a saline suspension of killed organisms equivalent to 6 sq. cm. of an 18 hour growth on glucose agar. In the following three intraperitoneal injections, given at 4 day intervals the number of organisms was doubled each time. The next four intraperitoneal injections were similar to the first four injections respectively, except that live organisms were employed. After a lapse of 2 weeks the equivalent of two loopfuls (in 1 cat, 1000 loopfuls) of an agar slant of live organisms was injected intravenously. The cats were bled out under anesthesia in intervals of from 1 week to 4 months.

None of the cats showed clinical or gross pathological evidence of disease. One cat showed several interstitial foci of necrosis with lymphocytic infiltration, large mononuclear cells and occasional binucleated cells in the heart muscle (Fig. 1). Four cats showed focal interstitial (in one case, perivascular, Fig. 2) accumulations of lymphocytes and mononuclear cells in the heart muscle. The organs were otherwise normal.

Swine

Two swine received each 9 injections of "rheumatic streptococci." The first intraperitoneal injections consisted of an amount of a saline suspension of killed

organisms equivalent of 12 sq. cm. of an 18 hour growth on glucose agar. In the following three intraperitoneal injections, given at 4 day intervals, the number of organisms was doubled each time. The next four intraperitoneal injections were similar to the first four except that live organisms were employed. After a lapse of 1 week, the equivalent of four loopfuls of an agar slant of live organisms was injected intravenously.

One swine developed slightly swollen knee joints which were neither red nor fluctuating, and a temperature of 104.6° 1 day after receiving the intravenous injection. No cultures were taken from these joints. In several days the joints subsided. 2 weeks after receiving the intravenous injection of organisms, the animal was bled out under anesthesia. All the organs including the joints were negative, macroscopically and microscopically.

The other swine also showed somewhat swollen knee joints the day following the intravenous injection of live organisms. There was no elevation of temperature. These joints were neither red nor fluctuating and subsided in a week. 1 week later the equivalent of 600 loopfuls of an agar slant of live organisms was injected intravenously. 1 month later the animal was bled out under anesthesia. The gross and microscopic findings were negative.

Sheep

One sheep received an intraperitoneal injection of an amount of saline suspension of killed organisms equivalent to 18 sq. cm. of an 18 hour growth on glucose agar. In the following three injections, given at 4 day intervals, the number of organisms was doubled each time. The next four intraperitoneal injections were similar to the first four injections, respectively except that live organisms were employed. Following a lapse of 2 weeks the equivalent of six loopfuls of live organisms was injected intravenously followed in 2 weeks by a similar injection of the equivalent of 600 loopfuls of live organisms. 1 month later the animal was bled out under anesthesia.

Following the third intraperitoneal injection of dead organisms the temperature rose to 107.4°F. 2 days later it fell to normal and continued so until the animal was bled out under anesthesia.

The only positive pathological finding was a few interstitial foci of lymphocytes and mononuclear cells in the heart muscle of the left ventricle.

Calves

Two calves received each 9 injections of "rheumatic streptococci." The procedure and doses were exactly the same as for the sheep in the previous experiment except that 3 weeks after the last intraperitoneal injection of live organisms the calves received each the equivalent of 600 loopfuls of live organisms injected intravenously. 4 months later both calves were bled out under anesthesia. The temperature remained normal throughout the experiment. The findings were the same as those of the sheep in the previous experiment.

Summary of Clinical and Pathological Findings in Group A

Out of the 24 rabbits, 12 guinea pigs, 5 dogs, 9 cats, 2 swine, 1 sheep and 2 calves used in this experiment, marked temperature elevation was noted only in the sheep and in one of the swine. Non-fluctuating arthritis which persisted for a short time only was observed in the swine. Most of the pathological lesions were found in the rabbits as follows: low grade, non-specific inflammatory, myocardial foci in 2, myocardial scarring in 4, purulent pleurisy and lobar or lobular pneumonia in 5, low grade pericarditis in 2, acute focal myocarditis in 1, and a non-bacterial thrombus in the mitral valve of 1. Six guinea pigs and five cats showed low grade, non-specific, inflammatory, myocardial foci. One dog showed a lobar pneumonia.

Group B

In this group 32 rabbits were employed. The object here was to produce in varying combinations thrombosis of a large vein with streptococcal invasion of the thrombus, blockage of the reticulo-endothelial system, poisoning of the heart muscle with Digitan and a continuous toxemia (if this were possible) by the injection subcutaneously of 15 cc. agar mass subsequently infected with "rheumatic streptococci."

The technic employed is described under "methods" (see above). The subgrouping of this experiment was as follows:

(a) 4 rabbits received Trypan blue, infected agar mass, thrombosis.
(b) 4 rabbits received Trypan blue, infected agar mass, thrombosis, Digitan.
(c) 4 rabbits received Trypan blue, thrombosis.
(d) 4 rabbits received Trypan blue, thrombosis, Digitan.
(e) 4 rabbits received thrombosis.
(f) 4 rabbits received thrombosis, Digitan.
(g) 4 rabbits received infected agar mass.
(h) 4 rabbits received infected agar mass, Digitan.

Each rabbit received in addition daily intravenous injections of 10 cc. broth culture of live pooled "rheumatic streptococci" consisting of Strains 1A-RG, 2A-JW, 3A-EB, 12A-LH, 26A-IS, 27A-SD, 28A-FB, 13A-R, 14A-R, 15A-R, 16A-R and 17A-R.

It may be said at the outset that there were no special differences in the findings of the various groups. The animals died or were killed in from 1 week to 5 months after the beginning of the experiment. Only seven rabbits survived over 2 months, at about which time they all showed swollen suppurating knee joints from which streptococci were cultured.

All but one of the rabbits had negative blood cultures. In most of the cases,

however, insufficient amounts of blood were obtained for culture because we did not wish to bleed directly from the heart. The positive culture was obtained from a rabbit who had suppurating knee joints and was bled out under anesthesia 3 months after the beginning of the experiments.

Another rabbit with suppurating knee joints was bled out and showed a bacterial (streptococcus) endocarditis involving the entire mitral valve, with acute focal suppurative myocarditis. The myocardium showed numerous streptococcus emboli in the blood vessels. The kidneys showed streptococcus emboli in the arterioles and glomerular loops. There was also a focal embolic glomerulonephritis not quite typical of the Loehlein lesion (Fig. 3). The spleen showed a large amount of brown pigment and an increase in large mononuclear cells.

Apart from this, three rabbits showed focal suppurative nephritis, two showed chronic interstitial nephritis, four showed scattered lymphocytic collections in the myocardium, one showed focal necrosis with calcification in the myocardium, four showed small scattered areas of scarring in the myocardium; one showed a chronic (lymphocytic) interstitial valvulitis of the mitral valve, one showed a suppurative pericarditis.

Group C

For these experiments there were employed 11 swine, 3 calves, and 2 sheep. The procedures in this group were so varied and extensive that it is desirable to record them in abstracted protocols (see appendix). Briefly stated, the animals in this group received numerous injections of agglutinated and unagglutinated "rheumatic streptococci;" of blood clot infected with "rheumatic streptococci;" of possible "virus" material represented by whole blood; plasma; serum; pericardial, pleural, and hydrocele fluid; and filtrates from tonsils, subcutaneous nodules, lymph nodes, and nasopharyngeal washings. This "virus" material was obtained from patients suffering of rheumatic disease (Table I). These materials were introduced through one or more of the following routes: intravenously, intraperitoneally, intracardially, and into the tonsils, trachea, or joints. As will be noted, the procedure was varied somewhat for the different animals.

Summary of Clinical and Pathological Findings in Group C

Swine

The experiments lasted from 1 week to 1 month. The temperature of some of the animals rose at times to 105.7°F., but this could not be correlated with any

of the other findings. None of these animals developed swollen joints or other discernible clinical manifestations. They were all bled out under anesthesia.

One swine (Swine 1) which had received an intracardiac injection, showed at autopsy an adherent pericarditis with acute and subacute inflammatory foci in the pericardium (Fig. 4) as well as in the superficial layers of heart muscle. No bacteria were found in the microscopic sections. Four showed occasional interstitial foci of lymphocytic and mononuclear cells in the heart muscle. Two showed a low grade chronic inflammation of the pleura associated with a fibrinous pericarditis. Two showed a small amount of scarring in the media of the aorta. One showed a mild interstitial valvulitis.

Calves

The three calves used in this experiment died 2 weeks, 5 weeks, and 3 months, respectively, after the beginning of the experiment. The temperature showed no unusual fluctuations. All the injections in these animals were done intravenously.

Two of the calves developed a suppurative arthritis of the knee joints from which streptococci were cultured. One of the calves developed the arthritis 3 weeks after the beginning of the experiment. At autopsy this calf showed an organizing synovitis. The findings were otherwise negative. The other calf (Calf 4) developed arthritis 10 weeks after the beginning of the experiment. At autopsy there was found a small warty vegetation in the left ventricle at about the middle of the interventricular septum (Fig. 5). The vegetation measured 2 x 3 mm., was raised 2 mm. above the endothelial lining, was smooth, with a broad base, and could be scraped off with the finger. Microscopic preparations with hematoxylin and eosin stains showed the vegetation to be covered by endothelium. The bulk of the vegetation showed purplish-pink staining, more or less hyaline material with a scattering of lymphocytes at its base. A few streptococci and Gram positive bacilli were found in the superficial layers of this thrombotic mass. The impression gained was that these organisms were post-mortem invaders. On the other hand, the heart muscle, spleen, and kidney showed many streptococcus emboli in the smaller vessels. A fibrinous pericarditis, a suppurative arthritis and a lobar pneumonia were the other findings in this animal.

The third calf (Calf 5) disclosed at autopsy a lobar pneumonia and a small warty nodule situated on the middle of the right posterior cusp of the aortic valve within the sinus pocket, 2 mm. below the free edge. The thrombotic mass measured 2 x 2 mm., was round and fairly smooth. On section it proved to be a typical streptococcus vegetation. The other organs were negative grossly. Unfortunately, however, the spleen and kidneys were lost before microscopical material was taken.

Sheep

The two sheep employed in this experiment died 9 and 10 weeks, respectively, after receiving the first injection. The temperature remained within normal limits.

One sheep received intravenous injections only. At autopsy there were disclosed lobar pneumonia and, microscopically, a few small interstitial foci of mononuclear cells in the myocardium. The other sheep (Sheep 3) received only intracardiac injections. After the third injection, 9 days after beginning of the experiments, positive blood cultures (streptococci) were obtained. This bacteremia remained until the death of the animal. 2 days before death, small petechiae which appeared to be white-centered were found in the conjunctivae of the animal. At autopsy there was found an organizing non-bacterial, fibrinous pericarditis, massive vegetative endocarditis of the aortic and mitral valves, fresh splenic infarction and bacterial emboli in the spleen, kidneys and heart muscle.

DISCUSSION

We have described a rather extensive attempt to induce rheumatic fever in animals by the use of streptococci isolated from patients suffering of rheumatic disease, as well as by the use of other material which might reasonably be supposed to contain the "virus" of the disease.

By consulting Table I it will be seen that many of the strains of streptococci were obtained from blood cultures in uncontaminated form. An analysis of the older up to the most recent literature on the subject brings out a striking similarity between the cultural characteristics of our organisms and many of those previously reported.

Some of the strains as well as "virus" material, it will be noted, were obtained from patients where a subsequent autopsy confirmed the diagnosis of rheumatic disease. The "virus" material was obtained in all instances from patients in whom the past history, present illness, and subsequent history left little doubt as to the correctness of the diagnosis. We desire to lay especial emphasis on our use of material from cases in which histological examination of biopsy material (subcutaneous nodules) as well as autopsy material made the diagnosis absolutely certain.

In contrast to this, much of the experimental work reported in the literature was carried on with streptococci isolated from contaminated sources, *e.g.*, tonsils, feces, post-mortem material, etc., or blood cultures from patients in whom the diagnosis of rheumatic disease was not confirmed by autopsy. It may, therefore, be argued that the equivocal results obtained by these workers were at least in many instances due to the fact that the etiological agent responsible for the disease (*i.e.*, the specific strain of streptococcus) was not employed.

Scattered references in the literature deal with observations on streptococci as secondary invaders in various conditions. Libman[33] has stressed this point for a number of years. In a recent article on general infections by bacteria, he states:

> As I have repeatedly emphasized, we now realize the fact that the anhemolytic streptococci constitute the most important secondary aerobic invaders with which the clinician has to reckon. They can be regarded as almost ubiquitous organisms, having been found in the blood in a great variety of conditions, in lymph nodes of various kinds removed during life, and quite regularly in post-mortem examination in organs studied in connection with etiologic investigations of several epidemic diseases.

The questionable value of the employment of streptococci obtained from post-mortem material has been further emphasized by the observations of Epstein and Kugel[32] in this laboratory. These workers, using routine autopsy material from non-rheumatic cases, have obtained streptococci of various types from perfectly normal valves in 40% of their cases, from heart muscle in 47%, from bone marrow in 67%, and from blood cultures in 79% of their cases.

It is obvious, therefore, that the finding of small numbers of such organisms in a vegetation, joint, pericardial cavity, subcutaneous nodule, etc., is by itself slim evidence as to their relation to the disease studied. In the absence of immunological reactions in the patients' blood against these organisms we must rely, at the present time, entirely upon the experimental production of the pathognomonic lesion of rheumatic disease, the Aschoff body, before concluding that the organism is responsible for the disease.

In the evaluation of our work we set for ourselves certain definite criteria which had to be fulfilled in order to establish the production of this disease. These criteria are the reproduction of (1) the Aschoff body, (2) non-bacterial pericarditis, (3) non-bacterial verrucous endocarditis. Of these, we held the Aschoff body to be, par excellence, the desideratum.

The production of migratory arthritis even of a non-bacterial type held for us but passing interest, first because it is not an essential concomitant of the disease in the human, secondly, because such joint conditions can be produced by a variety of agents. It is in the recognition of the experimentally produced Aschoff body that most

of the work in the past has been uncritical. For, whilst it is well known that the Aschoff body in the human heart is somewhat pleomorphic, one is reassured of his diagnosis of this lesion in the human heart even when it occurs in its less characteristic form, by the almost invariable coexistence of verrucous endocarditis, the less frequent occurrence of fibrinous non-bacterial pericarditis, the pathognomonic subcutaneous nodules and the long studied and fairly characteristic clinical course, together with the electro-cardiographic signs. One is further aided by the extensive knowledge of the pathology of the human heart where the tremendous fund of data gathered has shown that there are very few lesions with which the Aschoff body in its most characteristic form can be confused to the critical eye.

This is obviously not the case with experimental animals. Miller[23] has shown that in rabbits and guinea pigs, focal perivascular accumulations of large polyhedral cells with single or multiple nuclei are not infrequently found as spontaneous lesions. This we have been able to confirm. The spontaneous lesions which may occur in other experimental animals are not well known and perhaps it is not superfluous to state that one must guard against confusing such lesions with those intended to be produced experimentally. Further, the rabbit, and possibly other animals, are peculiarly prone to the formation of multinucleated cells in collections somewhat resembling Aschoff bodies following the injection of a variety of toxic substances. Fig. 6 shows such a lesion frequently produced in the rabbit's myocardium by the injection of an organism from the hemorrhagic septicemia group. This illustration can almost be superimposed upon one appearing in a recent publication from Small's laboratory by Belk and Jodzis.[22] These investigators produced this lesion by injections of "*Streptococcus cardio-arthritidis*" in rabbits and make the unequivocal statement that it is an Aschoff body in spite of the fact that they describe the finding of calcium deposition with giant cells in the heart.

Fig. 4 shows foreign body giant cells and polyhedral cells, with red staining protoplasmic granules as shown with the Unna Pappenheim stain, produced in the pericardium of Swine 1 in Group C. No bacteria were found in the preparation. Such a lesion can be easily confused with an Aschoff body to one who is not thoroughly familiar with the protean appearances of the latter.

With our associate, Dr. Benjamin Sacks, we submit the following structure as constituting an Aschoff body. For further details as to localization, Sacks'[34] excellent review may be consulted:

The Aschoff bodies consist essentially of focal collections of characteristic large cells belonging to the family of histiocytes, forming nodules of variable size and shape, those present in the myocardium being located in the interstitial connective tissue, in close relation as a rule to the coronary arterioles, and in the subendocardial tissue not in close proximity to blood vessels. The nodules are rounded, oval, globular, fusiform, spindle-shaped or entirely irregular in outline and save in very exceptional instances are not large enough to be seen with the naked eye. The interstitial nodules develop in the adventitia or peri-adventitial tissue of the coronary arterioles, or at some distance away from the vessel. The nodule may approach the vessel at only one point in its circumference or may spread out in both directions until it surrounds half or even the whole of the circumference. The close proximity of the nodule to the vessel may lead to compression of its lumen, especially when there are two nodules at opposite poles of the circumference. The interstitial connective tissue about the nodules is often edematous and some of the surrounding muscle fibers may undergo degeneration.

The predominating and essential cell of the Aschoff body is a large, polygonal element of variable size and shape, containing one or more nuclei. The cytoplasm, which has an indefinite, often ragged, limiting membrane, is rather homogeneous, non-vacuolated, not distinctly granular and in hematoxylin-eosin sections is distinctly, though not deeply *basophilic*. The nucleus may be ovoid or polymorphous. The former is somewhat vesicular and exhibits a *sharply defined nuclear membrane* with one or more nucleolus-like structures. The polymorphous type of nucleus is generally dark staining, large and occupies a relatively large part of the cell. The cytoplasm when stained by the Unna Pappenheim method with methyl green-pyronin assumes a distinctive brilliant red color, but this tinctorial property is shared by other cells (young cells) to such an extent that it is but rarely, if ever, useful for purposes of identification. The cells do not contain detectable phagocytosed material and no microorganisms or inclusion bodies have thus far been discovered. The multinucleated cells contain two to seven nuclei and in rare instances even more, but the staining properties of these cells are identical with those having a single nucleus. The multinucleated cells differ from the Langhans cells of tuberculosis in the central arrangement of their nuclei and resemble to a certain extent the Dorothy Reed cells of Hodgkin's disease. Their number in the Aschoff nodules is variable; at times they are few and at other times they may comprise as many as half of the characteristic cells. Mitoses have not been encountered. Intermingled with the most peripheral of the large cells and especially at the margins of the nodules there may be a variable number of other types of cells, including polymorphonuclear leucocytes, lymphocytes and plasma cells. At times these cells may be quite numerous.

In the fresher nodules it is common to find the characteristic cells arranged about a central zone of necrotic substance containing little or no fibrin. As the Aschoff bodies grow older, the cells become elongated and lose their characteristic appearance. Fibroblasts grow in among the cells and finally replace them. *Ultimately, the arteriole about which nodules have developed becomes surrounded by an oval or circular band of connective tissue which gives the interstitial tissue in rheumatic infection of the myocardium a very characteristic appearance.* In recurrent or chronic cases, new nodules may develop in such periarterial scars.

No description of the Aschoff bodies is complete without emphasing the extraordinary variability in the number, size, localization and appearance of these lesions, some of these peculiarities already having been referred to. At times the myocardium may be fairly riddled with large nodules accompanied by severe disorganization of the adjacent muscle fibers and what appears to be edema of the entire myocardium. In some cases, sheets of the characteristic cells may be found in localized areas of myocardium or pericardium or invading the valves from the rings. In the endocardium of the left auricle which is often the seat of a gross lesion, the Aschoff bodies are often located in rows about a necrotic collagen or elastic tissue fiber, the nuclei being perpendicular to the fibers or they may be located more deeply in the endocardium or subendocardium in the neighborhood of an irregular area of necrotic tissue, in both cases at some distance away from arterioles.

For the identification of Aschoff bodies, chief emphasis should be laid upon the presence of these nodules in the periarterial tissue of the myocardium with the subsequent development of circular perivascular fibrosis, the tendency for the component cells to arrange themselves about necrotic material (collagen?) and the morphology and tinctorial properties of the characteristic mononuclear and multinucleated cells, especially the basophilic cytoplasm and the dark nucleus with its sharply defined nuclear membrane and nucleoli.

It is precisely because there is here room for individual interpretation that we have insisted on the other two criteria as well, namely, the non-bacterial verrucous endocarditis and the non-bacterial pericarditis.

A number of reports on the experimental reproduction of rheumatic endocarditis describe what is obviously vegetative endocarditis (so-called malignant endocarditis). Apart from the fact that a great variety of organisms can produce this condition (Menzer,[35] Cole,[7] Horder,[23] Thalhimer and Rothschild[25]), we can under no circumstances accept such a lesion as of rheumatic origin.

It might be contended that we are too stringent in our postulates, that possibly species other than human may present an entirely different pathological reaction to the same agent or, indeed, may not

be susceptible to the disease. While such a state of affairs would be highly regrettable, it does not justify the assumption that vegetative endocarditis is verrucous endocarditis or that focal inflammatory accumulations in an animal's myocardium are the equivalent of Aschoff bodies in the human. And yet just such assumptions have been made.

Following this preliminary discussion there is little further we need say in the appraisal of our work. We have found in 24 of our animals focal accumulations of inflammatory cells—at times closely resembling Aschoff bodies and yet so distinct from the latter that we can unconditionally state that we have *not* reproduced the classical Aschoff body. We have also found pericarditis in 8 animals, arthritis in 12 and vegetative endocarditis in 4. Furthermore, we feel reasonably certain that these lesions were not spontaneous occurrences. Indeed, in one case (Fig. 5), a lesion was produced in the outflow tract of the left ventricle showing a remarkable resemblance to a rheumatic vegetation. This animal, however, showed embolic bacterial lesions in the kidney and spleen. In short, whilst many lesions have been produced, we cannot state that we have induced rheumatic disease in animals by the use of streptococci nor by other materials obtained from human cases of this disease; this, despite the fact that we have employed a large number of animals, a variety of species and a variety of procedures far greater than that hitherto reported in the literature. To this we must add the statement that we have also employed the streptococcus in conjunction with possible "virus" material in the same animals with equally unsuccessful results.

Before drawing final conclusions, we must emphasize that this work does not represent an attempt to disprove the streptococcal theory of the etiology of rheumatic fever. It is certainly possible that our negative results may have been due to the fact that despite the complexity of our experiments and our deliberate attempts to sensitize our animals to the streptococcus, we failed to induce the necessary receptivity of the tissues, (Swift *et al.*,[9] Semsroth and Koch[39]) if indeed, such a state of receptivity is an essential prerequisite.

As we have indicated, however, quite positive conclusions have been reached by previous workers. Inasmuch as we have repeated much of the work reported in the past, and have amplified these experiments

in many ways and on an extensive scale, it seems justifiable to point out that rheumatic fever, recognizable as such, has not been reproduced, and to conclude on the basis of such work that either the streptococcus is not the etiological agent of rheumatic fever, or that the species of animal employed are not susceptible to this disease—at least under the conditions of these experiments—or finally that rheumatic manifestations in the species employed differ markedly from those found in the human. Until it is proved that the last of these assumptions is correct, one is not justified in making it.

CONCLUSIONS

Experiments have been described in which we attempted to reproduce in animals the lesions characteristic of rheumatic fever in the human. A large number of animals representing 7 species was employed. Among other materials, streptococci isolated in pure culture from the blood of rheumatic patients (proved to be so by biopsy or by autopsy) as well as whole blood, plasma, serum, pericardial, pleural and hydrocele fluid, filtrates from tonsils, subcutaneous nodules, lymph nodes, and nasopharyngeal washings obtained from such patients were used in a variety of combinations and with a number of procedures calculated to predispose the animal to the disease.

A discussion is given of the criteria whose fulfillment is essential for the establishment of the experimental production of rheumatic disease in animals.

Judged by these criteria, we have failed to reproduce the disease. This conclusion, we believe, holds true for all the work thus far reported in the literature.

APPENDIX

Protocols of Group C

(The following abbreviations are employed: pld = pooled; IV = intravenously; IP = intraperitoneally; IT = intratracheally; cit. & def. = citrated and defibrinated; NP = nasopharyngeal mucous membrane; agg = agglutinated.)

Swine 1.—(10/30/25) 10 cc. pld Mass 1,* IV. (11/2/25) femoral vein crushed,

* Mass 1 consists of pooled 1A-RG, 2A-JW, 26A-IS, 27A-SD, 28A-FB.

10 cc. pld Mass 1, IP. (11/5/25) 10 cc. pld Mass 1, into tonsils. (11/6/25) 10 cc. pld Mass 1 into tonsils, and 10 cc. IV. (11/9/25) 10 cc. pld 2A-JW, 21A-VS, 35A-MS, IV. (11/10/25) blood culture taken (streptococci), 10 cc. pld 2A-JW, 27A-SD, 21A-VS, 35A-MS, IV. (11/11/25) 10 cc. pld 24A-AW, 28A-FB, 27A-SD, 2A-JW, IT. (11/17/25) 10 cc. pld 1A-RG, 28A-FB, 24A-AW, 26A-IS, 35A-MS, into heart. (11/18/25) Swine very sick, can scarcely stand. 10 cc. pld 27A-SD, 2A-JW, 22A-YL, into joints. (11/30/25) Bled out under anesthesia. (For findings see page 53.)

Swine 4.—(12/9/25) 15 cc. pld 2A-JW, 26A-IS, 3A-EB, 1A-RG, IP. (12/12/25) 15 cc. culture of streptococci recovered from Swine 1, IP. (12/16/25) 3 cc. pld 3A-EB, 32A-AK, 23A-MB, 33A-HH, IV. Animal died. Organs grossly negative. Auricular muscle lymphocytic interstitial myocarditis.

Swine 5.—(12/8/25) blood culture taken (negative). 5 cc. cit. & def. blood (32A-AK), IV, and 25 cc., IP. (12/9/25) 10 cc. serum (32B-AK), IV. (12/16/25) 1 cc. cit. & def. blood (19A-RB), IV, 60 cc., IP. (12/28/25) 20 cc. cit. & def. blood (18B-VF), IP. (12/31/25) Blood culture taken (negative.) Bled out under anesthesia. Findings negative.

Swine 6.—(12/20/25) Blood culture (negative). Superficial vein crushed. 10 cc. pld 1A-RG, 2A-JW, 12A-LH, 3A-EB, 26A-IS, IV, 5 cc. into each tonsil, 5 cc. into NP, 1 cc. into each ankle joint, and 25 cc. 23A-MB, IP. (12/21/25) 10 cc. agar mass infected with pld Mass 1, IP. (12/22/25) 5 cc. pld Mass 1, IT. (12/23/25) 10 cc. pld Mass 1, IV. (12/24/25) Bled out under anesthesia. Auricle shows occasional perivascular mononuclear accumulations. Otherwise findings negative.

Swine 7.—(12/20/25) Blood culture (negative). Large superficial vein crushed. 15 cc. pld Mass 1, IV. 5 cc. into each tonsil and NP, 1 cc. into each of 4 joints. 25 cc. 23A-MB, IP. (12/21/25) 10 cc. agar mass infected with pld Mass 1, IP. (12/22/25) 5 cc. pld Mass 1, IT. (12/23/25) 7 cc. pld Mass 1, IV. (12/28/25) 20 cc. cit. & def. blood (18B-VF), IP. (12/29/25) 20 cc. pld 12A-LH, 3A-EB, 2A-JW, IV, and 30 cc. IP. 15 cc. agar mass infected with these cultures, IP. 4 cc. post-mortem pericardial fluid (18A-VF) ground up with blood clot of Swine 7, IV. Swine developed edema of lung. (12/31/25) 17 cc. cit. & def. blood (37B-JR), IP. (1/2/26) Bled out under anesthesia. Temperature of this swine rose to 106°F. on 12/26/25. Only findings are organizing peritonitis.

Swine 8.—(12/20/25) Blood culture (negative). Large superficial vein crushed. 10 cc. pld Mass 1, IV, 1 cc. into each of 2 joints, 5 cc. into tonsil and NP, 25 cc. 23A-MB, IP. (12/21/25) 10 cc. agar mass infected with pld Mass 1, IP. (12/22/25) 5 cc. pld Mass 1, IT. (12/23/25) 10 cc. pld Mass 1, IV. (12/28/25) 20 cc. cit. & def. blood (18B-VF), IP. (12/29/25) Swine died. Organizing fibrinous pericarditis and peritonitis. Scattered interstitial collections of lymphocytes and monocytes in pericardium.

Swine 10.—Treatment similar to Swine 8. (1/7/26) Bled out under anesthesia. Blood culture (negative). Fibrinous and mononuclear pericarditis.

Swine 12.—Treatment the same as Swine 8. (1/14/26) Bled out under anesthesia. A few scattered mononuclear interstitial foci. Lymphocytic interstitial valvulitis of mitral valve.

Swine 13.—Treatment the same as Swine 8. (12/29/25) Bled out under anesthesia. Blood culture (negative). Negative findings.

Swine 15.—Treatment the same as Swine 8. (1/18/26) Bled out under anesthesia. Blood culture (negative). Fibrinous pericarditis and pleuritis. Focal lymphocytic and monocytic interstitial collections in myocardium.

Calf 1.—(6/17/26) 50 cc. pld "R" cultures,* IV. (6/18/26) Left femoral vein painted with 20% silver nitrate. 15 cc. ground up blood clot from Calf 2 previously incubated for 24 hours with 1 cc. pld "R" cultures, IV. 40 cc. pld "R" culture, IV. (6/23/26) 50 cc. pld Mass 1 and 2,† IV. (6/24/26) 50 cc. pld Mass 1 and 2, IV. (6/25/26) 10 cc. nasal washings (37A-JR) incubated with 2 cc. pld "R" cultures for 24 hours, IV. 40 cc. Mass 1 and 2 agg with homologous rabbit serum and washed, IV. (6/26/26) 2.5 cc. "R" cultures, 22.5 cc. Mass 1 and 2, and 25 cc. agg Mass 1 and 2, IV. (6/28/26) 30 cc. pld "R" cultures, IV. Repeated for following 2 days. (7/2/26) 20 cc. nasal filtrate (37A-JR) and 40 cc. pld "R" cultures, IV. (7/7/26) 30 cc. hydrocele fluid (30A-MB) and 20 cc. agg "R" cultures, IV. (7/9/26) Blood culture (negative). 20 cc. ground up blood clot from Calf 1 which had been incubated over night with 20 cc. pld Mass 1 and 2, IV. (7/10/26) 20 cc. infected blood clot (as above), 20 cc. hydrocele fluid (30A-MB), and 5 cc. tonsillar filtrates (31B-AW), intravenously. (7/15/26) 24 cc. serum (31C-AW) and 5 cc. "R" cultures, IV. Calf died. Findings completely negative.

Calf 4.—(6/30/26) Collodion sacs containing respectively, 3A-EB, 13A-R, 14A-R, 26A-IS, and pld "R" cultures in broth implanted IP. Left femoral vein painted with 20% silver nitrate, 50 cc. pld "R" cultures, IV. (7/7/26) 50 cc. agg "R" culture, IV. Blood culture taken (negative). (7/10/26) 25 cc. infected blood clot (similar to Calf 1), and 5 cc. tonsil filtrate (31B-AW), IV. (7/15/26) Treatment same as Calf 1. (7/24/26) 10 cc. plasma and buffy layer (31A-AW), and 10 cc. 25A-RG, IV. (7/28/26) 10 cc. filtrates of subcutaneous nodules (40A-AL), 10 cc. filtrates of lymph nodes (40B-AL), 10 cc. pld Mass 1 and 2, and 10 cc. agg Mass 1 and 2, IV. (7/30/26) 10 cc. agg pld "R" cultures and 5 cc. plasma and buffy layer (39A-EZ), IV. (7/31/26) 5 cc. pericardial fluid (29A-MM), 5 cc. tonsil filtrate (34A-JR), and 5 cc. pld "R" cultures, IV. (8/3/26) 10 cc. pericardial fluid (29A-MM), 5 cc. serum (36A-HM), and 5 cc. pld "R" strains, IV. (8/7/26) Right knee joint markedly swollen. (8/19/26) 15 cc. pld "R" cultures, 5 cc. pericardial fluid (29A-MM), 5 cc. lymph node filtrates (29B-MM), IV. (8/26/26) 30 cc. agg Mass 1 and 2, and 5 cc. pericardial fluid

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* "R" cultures consist of 13A-R, 14A-R, 15A-R, 16A-R, 17A-R.

† Mass 2 cultures consist of pooled 2A-EB, 12A-LH, pooled "R" cultures, 2A-JW, and strains recovered from Swine 1.

(29A-MM), IV. (9/4/26) 40 cc. pld "R" cultures and 10 cc. tonsil filtrate (38A-MP), IV. Swelling gone from knee joint. (9/11/26) 30 cc. pld "R" cultures and 10 cc. tonsil filtrate (20A-SY), IV. (9/17/26) Similar to Sheep 1 on 9/16/26, IV. (9/22/26) Capsules removed from peritoneal cavity and cultured on blood plates and veal broth. (9/24/26) 50 cc. pld rheumatic capsule strains from Calf 4, IV. (9/25/26) Calf very sick. (9/27/26) Abdominal wound broke open, dressed. (9/26/26) Calf died. (For findings see page 53).

Calf 5.—Treatment the same as Calf 4, except that no capsules were implanted IP. No swollen joints developed. (8/7/26) Calf died. (For findings see page 53).

Sheep 1.—(7/29/26) Collodion capsules, containing respectively individual "R" strains, 3A-EB, 26A-IS, organisms obtained from blood of Swine 1, and 2A-JW, implanted IP. 30 cc. pld Mass 1 and 2, IV. (8/18/26) 20 cc. pld Mass 1, 5 cc. each of pericardial fluid (29A-MM) and lymph nodes filtrate (29B-MM), IV. (8/26/26) 20 cc. agg Mass 1 and 2, and 5 cc. pericardial fluid (29A-MM), IV. (9/4/26) 25 cc. agg Mass 1 and 2, and 5 cc. tonsil filtrate (38A-MP), IV. (9/11/26) 25 cc. agg Mass 1, and 5 cc. tonsil filtrate (20A-SY), IV. (9/15/26) IP capsules removed and contents cultured on blood slants and mutton broth. (9/17/26) Capsule strains emulsified with 2% agar, 20 cc. injected IV. (10/2/26) 50 cc. pld capsule strains from Sheep 1, IV. (10/8/26) Stitches of wound infected. Capsule cultures emulsified with gum Tragacanth (5% emulsion), 25 cc. injected IV. 1 hour later sheep died. A few interstitial mononuclear foci, otherwise *findings negative.*

Sheep 3.—(10/15/26) 50 cc. agg pld capsule strains from Sheep 1, intracardially. This was repeated on 10/19/26, and on 10/23/26. (10/25/26) Blood cultures show Gram positive cocci. (10/26/26) 80 cc. agg pld capsules strains of Sheep 1, and 10 cc. tonsil filtrate (20A-SY), intracardially. (10/29/26) 80 cc. agg pld capsule strains from Sheep 1, intracardially. Blood culture (streptococci). (11/4/26) 30 cc. agg pld capsule strains from Sheep 1, intracardially. (11/9/26) Similar to 11/4/26, but 50 cc. injected. (11/12/26) Similar to 11/9/26. Blood culture (Gram positive cocci). (11/19/29) Blood culture (streptococci). (12/7/26) 5 cc. plasma and buffy layer (10B-IS), 40 cc. agg pld capsule strains from Sheep 1, and 5 cc. 7A-MB, intracardially. (12/29/26) Sheep died. (For findings see page 54).

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Area of focal necrosis with lymphocytes and large mononuclear cells in cat's myocardium (Group A).

FIG. 2. Perivascular foci of lymphocytes and large mononuclear cells in cat's myocardium (Group A).

PLATE 2

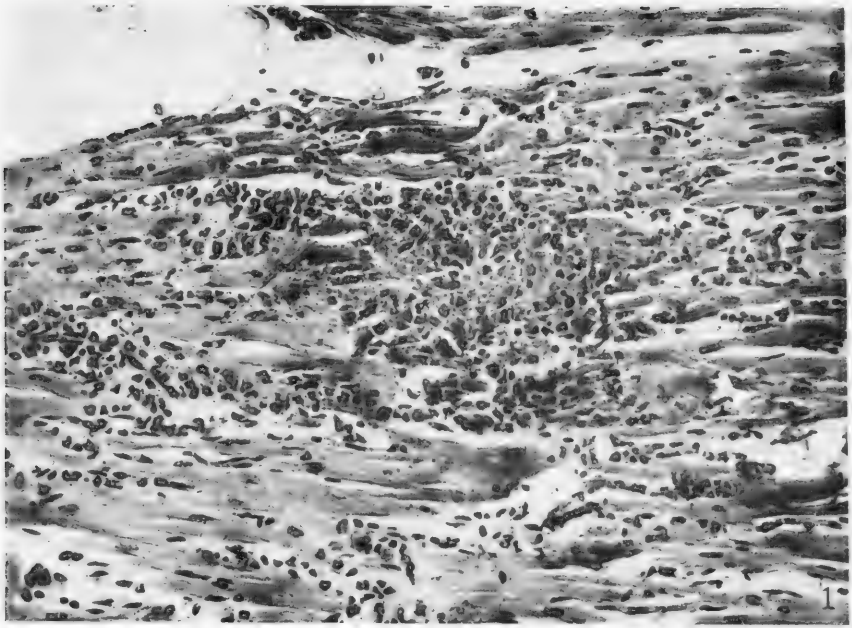
FIG. 3. Focal embolic glomerulo-nephritis in kidney of rabbit (Group B).

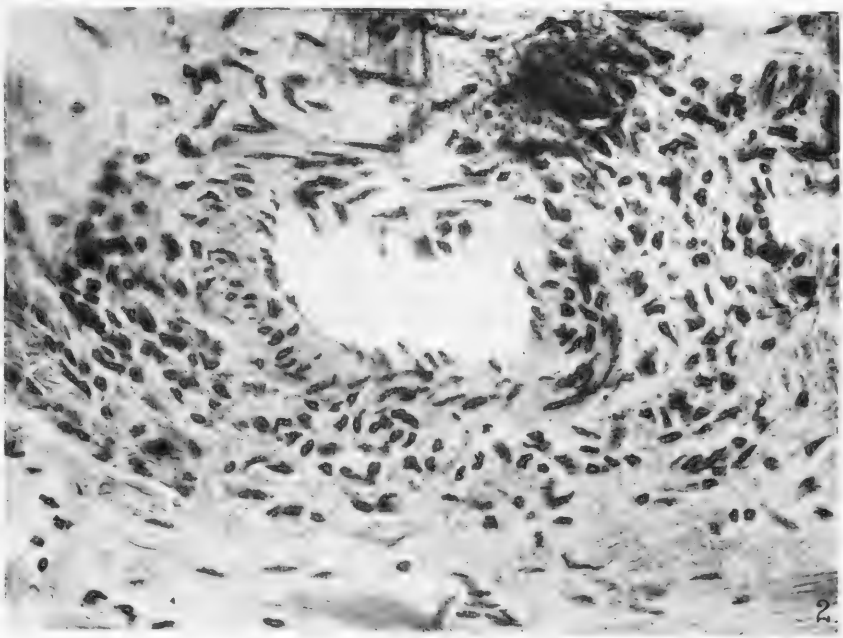
FIG. 4. Nodule in pericardium of Swine 1 (Group C), showing collection of large mononuclear cells and giant cells.

PLATE 3

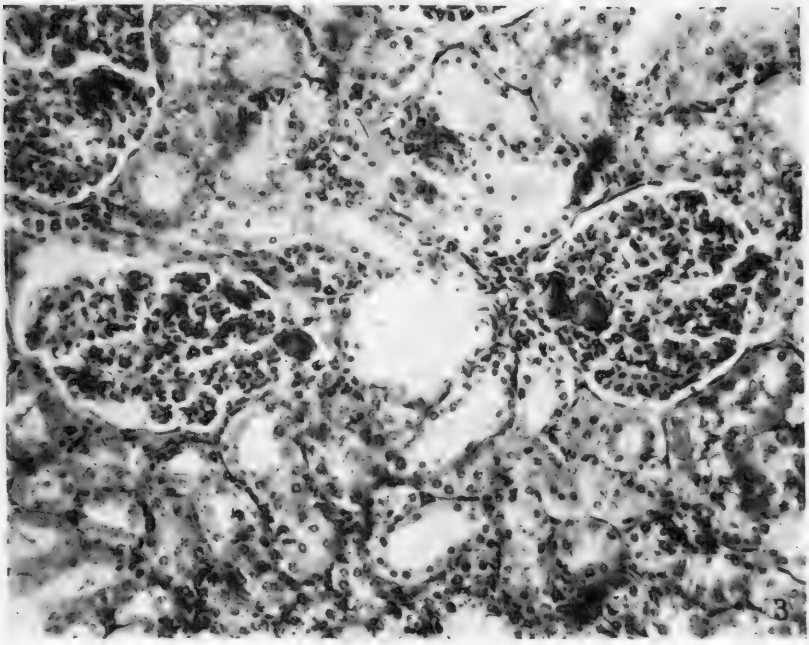
FIG. 5. Verrucous nodule in left outflow tract (interventricular septum) of Calf 4 (Group C).

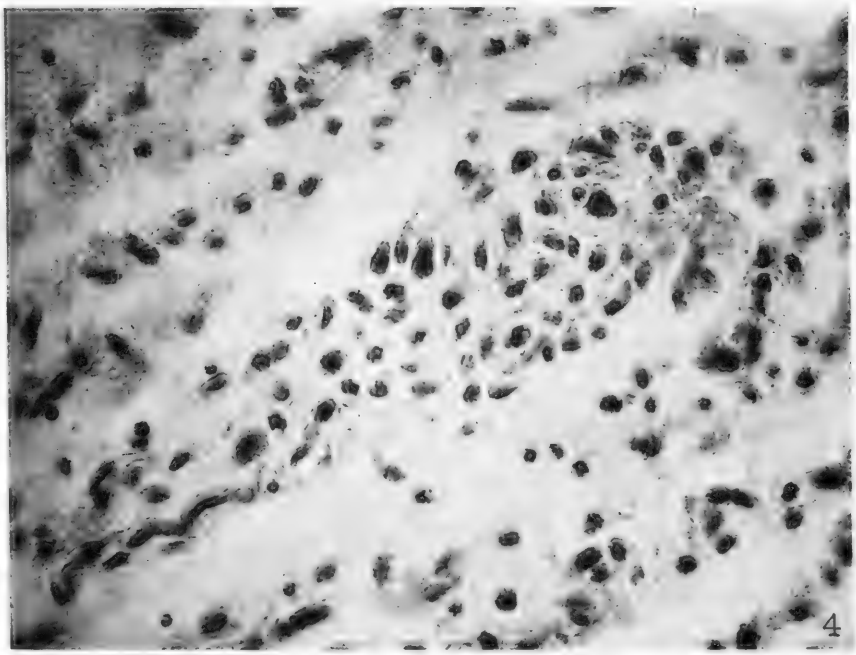
FIG. 6. Foreign body giant cells and calcium deposit in left auricle of rabbit.





(Gross *et al.*: Rheumatic fever in animals)





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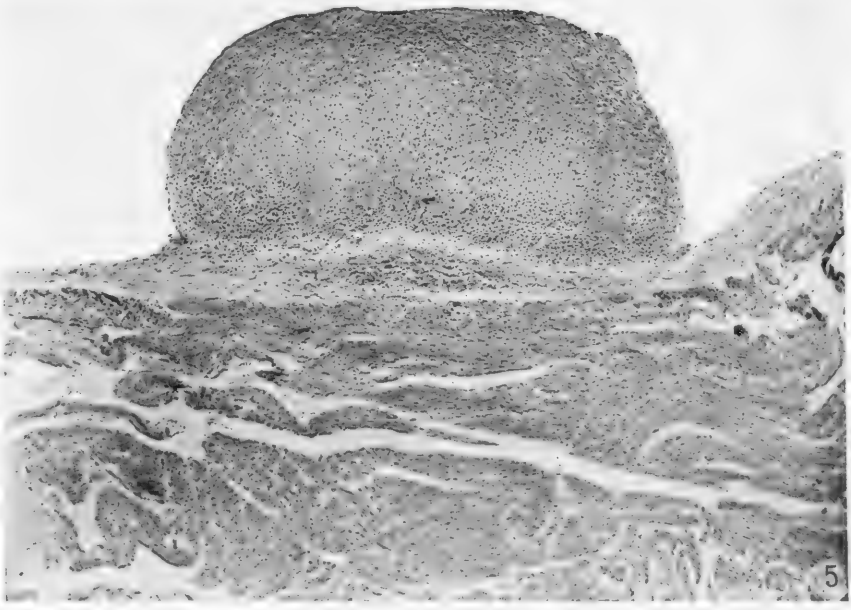

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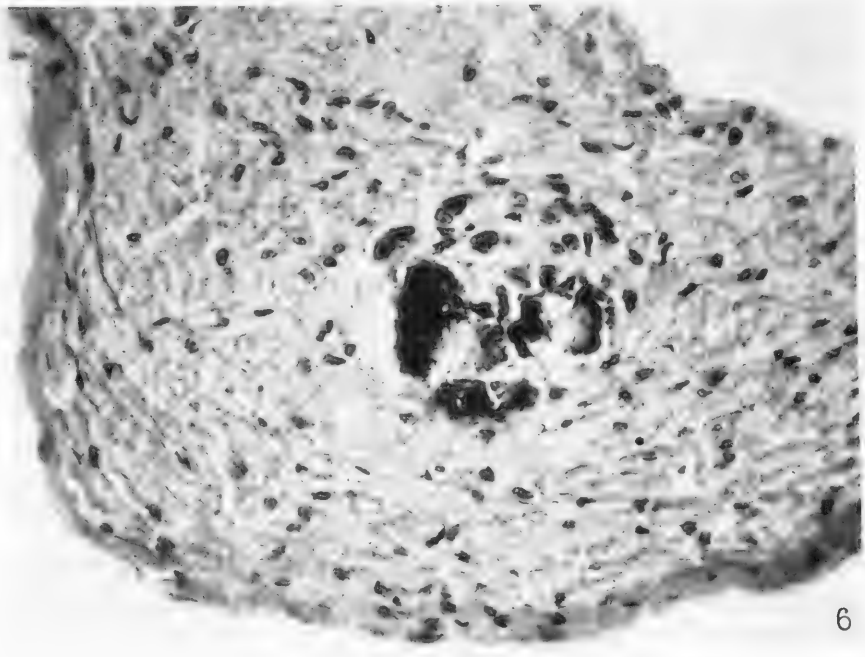

6

(Gross *et al.*: Rheumatic fever in animals)

ON THE RELATION OF BACTERIA TO SO-CALLED "CHEMICAL PNEUMONIA"

By A. R. KOONTZ, M.D., and M. S. ALLEN

(*From the Medical Research Division, Chemical Warfare Service, U. S. Army*)

PLATES 4 AND 5

(Received for publication, April 22, 1929)

I. HISTORICAL

The pneumonia resulting from inhalation of irritating substances has been said to be caused purely by the chemical agents inhaled, and not to be due to the action of bacteria.

Delafield, Prudden and Wood (1) state that "the inhalation of irritating gases, especially chlorine, ammonia, nitrogen tetroxide, bromine, and a large number of the corrosive organic products now used as destructive agents in war, gives rise to a characteristic type of pneumonia due solely to the chemical action and not necessarily accompanied by bacterial infection, although this may occur."

Wood (2) reports a case of pneumonia occurring from breathing nitric oxide fumes which resulted fatally. The pneumonia was lobular in type, with desquamation of mucous membrane of trachea and bronchi, cellular plugs in the bronchi, and areas of consolidation around the smaller bronchi. The alveoli in the consolidated areas were "filled either with a transparent albuminous mass, or with fibrin containing a few leucocytes, or with desquamated alveolar epithelium, ... Throughout, many alveoli contained red blood corpuscles." Various staining methods showed no organisms. Wood reviewed the literature and found two cases reported by Loeschcke (3), in which a search for bacteria in sections had failed to reveal their presence. Pneumonia induced in animals with nitrogen tetroxide (N_2O_4) fumes was characterized by edema, fibrinous exudate, red and white cells and desquamated alveolar epithelium. Cultures were sterile.

Karsner (4) and Karsner and Ash (5) have shown that rabbits exposed to atmospheres rich in oxygen for 24 to 48 hours, develop a fibrinous broncho-pneumonia in which edema and desquamation of the alveolar epithelium play a prominent part. They think that "the absence of any well marked leucocytic infiltration in the pneumonia area and the absence of demonstrable leucocytosis in the circulating blood point toward a pneumonia of irritative rather than of bacterial origin." However, bacteriological studies of the lungs are not reported.

Wollstein and Meltzer (6) in the course of some intra-bronchial insufflations found that certain salt solutions caused pneumonia. 2 per cent sodium chloride solution, sodium sulfate, magnesium sulfate, and magnesium chloride solutions produced small patches of broncho-pneumonia. Cultures showed no growth. Mercuric chloride, 1–10,000 dilution, caused hemorrhage, edema and thrombi; no bacteria grew. In another series of experiments (7) they caused broncho-pneumonia by the injection of chloramine-T and Dakin's solution. The pneumonias produced by these solutions were characterized by the absence of fever or appearance of illness. There is no record of bacteriological studies. Kline and Meltzer (8) found that aleuronat and starch caused consolidation resembling that caused by a virulent pneumococcus. Egg yolk and lecithin caused a lobar pneumonia like that caused by a virulent pneumococcus. Cultures were sterile in the majority of their experiments. Wollstein and Meltzer (7) state that "the consolidations of the lung produced by chemical substances differ from infectious pulmonary inflammations only in their sterility."

On the other hand, Winternitz, Lambert, Jackson and Smith (9) find that after chlorine poisoning organisms "that normally inhabit the mouth of the dog find their way into the bronchi and lungs of the dog shortly after gassing and remain there for a long time in animals that survive the acute period." They show "that the pneumococcus and a small Gram-negative hemoglobinophilic organism, normal inhabitants of the dog's mouth, can be cultivated from the lung as early as one-half hour and as late as four days after gassing." Similarly, Winternitz, Lambert and Jackson (10), in studying the bacteriology of pneumonias following gassing with phosgene, found that the bacteriological findings were much the same as in cases of pneumonia in non-gassed dogs.

II. EXPERIMENTAL

In view of the high incidence of pneumonia in the lungs of animals experimentally gassed with toxic compounds in the toxicity work, Medical Research Division, Edgewood Arsenal (11), ample opportunity was offered for the study of the types of pneumonias so caused, and of the incidence of bacteria in these pneumonias.

As cultures of the lungs had not been made at the time of autopsy, the lung sections were stained with Goodpasture-MacCallum stain in order to study the bacteria present. This had the advantage over cultures of giving the number of bacteria present, and showing their position in regard to the lung framework.

The animals studied had been placed in the ice-box as soon as they were found dead, and the autopsy performed as soon as possible thereafter. In studying the sections from these animals the time elapsing between death and autopsy was considered, but this was not found to affect the number of bacteria present when the autopsy was performed within a reasonable limit.

III. METHODS

The tissues were fixed in Zenker-formol solution and embedded in paraffin. The lungs were fixed by running the fixing fluid into the trachea, and then immersing them in the fluid for 24 hours before any sections were taken, as this has been found necessary to preserve the delicate structure and prevent artifacts (12). The sections were stained with hemotoxylin and eosin to study the types of pneumonia, and with Goodpasture-MacCallum stain to study the bacteria present.

IV. DISCUSSION

The types of pneumonia and the bacteria present were studied in the lungs of dogs gassed with mustard gas, methyldichlorarsine, lewisite, phosgene, and some miscellaneous gases.

The significance of the number of bacteria present was considered in reference to:

A. The kind of gas inhaled.
B. The type of pneumonic exudate.
C. The temperature of the animal during illness.
D. The length of time between death and autopsy.
E. The survival after gassing.
F. Bacteria in non-gassed lungs.
    1. In normal lungs.
    2. From pneumonia experimentally caused.
    3. From cases of pneumonia occurring spontaneously in dogs.

A. Relation of Bacteria to the Kind of Gas Inhaled

1. Mustard.—The lungs of mustard gas poisoning show the following lesions in the order of their frequency: hemorrhage; atelectasis; edema; and exudate consisting of epithelial cells, leucocytes, and mononuclear cells, but very little fibrin. In a majority of cases epithelial cells and polymorphonuclear leucocytes are present in large numbers. In a few cases epithelial cells and mononuclears prevail.

Of thirty-two cases studied, nine showed a few bacteria, sixteen showed many and seven a large number. There was no correlation observed between the type of exudate and the number of bacteria.

Table I gives a few typical cases.

2. Methyldichlorarsine.—The pneumonia in methyldichlorarsine poisoning consists of an exudate in which epithelial cells and mononu-

clears predominate. In only a few cases are they out-numbered by polymorphonuclear leucocytes.

In the lungs of eighteen cases, twelve showed few bacteria, two showed many, and four large numbers.

TABLE I

Results Showing Number of Bacteria in a Few Typical Mustard Lungs

General condition of lung	Pneumonic exudate	Bacteria
Hemorrhage Atelectasis	None	Few
Solid with exudate	Leucocytes	Very few
Nearly normal on recovery from gassing	Organized areas and desquamating epithelial cells	Large numbers
Hemorrhage Atelectasis	Leucocytes, epithelial cells	Few
Congestion Hemorrhage	Epithelium, leucocytes	Many
Congestion Hemorrhage Edema	Bronchial exudate	Many
Fibrin, edema	Leucocytes, epithelial cells	Many chains
Necrotic areas	Leucocytes, mononuclears	Enormous numbers in necrotic areas

No correlation was seen between the number of bacteria and the type of exudate, although this gas as a whole showed a small number of bacteria.

Table II gives the summary of findings for methyldichlorarsine in a few typical cases.

3. Lewisite.—A leucocytic exudate prevails in lewisite lungs. In practically all acute deaths leucocytes are predominant with epithelial cells and mononuclears present in much smaller numbers.

Of twenty-seven cases, seventeen showed a few bacteria, three

showed no bacteria, seven had many. No cases showed large numbers of bacteria. The lungs from this series showed fewer bacteria than any other.

4. Phosgene.—The first lesion in phosgene poisoning, and the one from which death usually occurs, is edema. If survival is prolonged

TABLE II

Results Showing Number of Bacteria in a Few Typical Methyldichlorarsine Lungs

General condition of lungs	Pneumonic exudate	Bacteria
Congestion Hemorrhage	Scattered leucocytes Epithelial cells Red blood cells	Few
Edema Hemorrhage	Red blood cells Leucocytes	Few, chiefly near bronchi
Hemorrhage Atelectasis Fibrin	Swollen epithelial cells	Few
Edema	Epithelial cells Mononuclears	Very few
Congestion	Leucocytes Few epithelial cells	Few Some intracellular
Fibrin Necrosis Hemorrhage	Leucocytes Few epithelial cells	Necrotic areas loaded with bacteria
Edema	Leucocytes Mononuclears Epithelial cells	Few Some intracellular

the edema fluid becomes infiltrated with leucocytes so that in the later deaths pneumonia is a common finding. The pneumonia is not peribronchial in character but is generalized throughout the alveoli as if arising from the alveolar walls. Leucocytes, mononuclears and epithelial cells are present. Most phosgene lungs in which an exudate was present, showed many bacteria.

B. Relation of Bacteria to Type of Exudate

Desquamated epithelium is conspicuous in pneumonias due to irritating gases but in only a few cases does it form the entire exudate. Usually the picture is that of broncho-pneumonia, with epithelial cells, mononuclears and polymorphonuclear leucocytes composing the exudate. Frequently the leucocytes out-number the other cells.

The pneumonia of gassed lungs resembles the broncho-pneumonia seen in non-gassed lungs. Sometimes it is peribronchial in type, sometimes it involves a lobule uniformly. Hemorrhage, atelectasis and perivascular and peribronchial edema are typical findings in gassed lungs.

A glance at Table III shows that bacteria are prevalent in all types of pneumonic exudates found in gassed lungs.

TABLE III

Results Showing Relation of Bacteria to Type of Exudate

Type of exudate	Few bacteria	Many bacteria	Large numbers
Leucocytes predominating	50%	30%	20%
Epithelial cells predominating	33%	47%	20%
Leucocytes and epithelial cells in equal numbers	54%	38%	8%

C. The Temperature of the Animal

As a general rule gassed dogs do not run noticeably high temperatures. This would seem to indicate an absence of bacterial infection, but the inference cannot be drawn, as no information is at hand as to what temperature dogs show when infection is known to be present. A dog's temperature varies so markedly from day to day and from hour to hour that it does not appear to be an index of the animal's condition.

The highest temperature seen in the series studied was 106.2°F. The exudate in this animal was purely leucocytic, but very few bacteria were found. Another dog with a temperature of 106°F., showed necrosis of the lungs and large numbers of bacteria. In a dog showing a temperature of 104.2°F., the exudate was leucocytic and many

bacteria were present, but on the other hand, in several cases showing many bacteria, the temperature was normal.

D. The Length of Time Between Death and Autopsy

There does not appear to be any relation between the time elapsing after death and the number of bacteria. Some of the cases showing the largest number of bacteria were autopsied very soon after death and some cases showing very few bacteria had been kept as long as 18 hours before autopsy was performed.

TABLE IV

Results Showing Number of Bacteria in Lungs of Dogs Surviving Varying Lengths of Time after Gassing

Hours of survival	No. of cases	Lungs	Bacteria
Less than 12	5	No exudate	Few
12	1	Leucocytes present	Few
12–24	6	Leucocytes and epithelium	Few 4, many 1, some 1
24–36	5	Leucocytes	Few 3, none 2
36–48	4	Leucocytes	Few 3, many 1
48–72	14	Leucocytes	Few 6, many 8
72–96	5	Leucocytes, 2 necrosis	Few 2, many intracellular 3
120	4	Leucocytes, 1 necrosis	Many 4, intracellular
140	5	Leucocytes, 2 necrosis	1 Few, 4 many
9 days	1	Necrosis	Many, intracellular
22 days	1	Leucocytes	Very many
9 weeks	1	Chronic exudate	None

The temperature at which the body was kept after death undoubtedly had more effect on the number of bacteria in the lungs, than did the length of time between death and autopsy.

No record of temperature is available but in view of the well preserved state of the cells it may be assumed that post-mortem degeneration was slight.

E. The Survival after Gassing

If the bacteria found in the lungs are present in a causal relationship, it would be expected that the early deaths from pneumonia would

show as many bacteria as the later deaths, or at least that bacteria would be present in considerable numbers in the early deaths. If the presence of the bacteria is merely casual, and due to the injured lungs offering a favorable field for growth, the number might be expected to be smaller in the early deaths and to show an increase as the time of survival is longer.

The cases were grouped and studied therefore with regard to the number of hours of survival (Table IV).

Animals dying in less than 12 hours showed no exudate in the alveoli but a bronchial exudate was frequently present. At 12 hours there was one case of well-developed pneumonia, and above 12 hours most cases showed pneumonia. Areas of necrosis were not observed

TABLE V

Summary of Table IV

Hours of survival	No. of cases	Bacteria
Less than 48 hours	21	None 2, few 16, many 3
48–72 hours	14	Few 6, many 8
72 hours–9 weeks	17	Few 3, many 14, many intracellular

in deaths below 72 hours. Above that they were frequent. Above 72 hours many of the bacteria were observed to be engulfed by the leucocytes. Below 72 hours very few intracellular bacteria were seen.

Tables IV and V show that there were more bacteria in the lungs of animals surviving 48 hours or longer, than in those of animals dying at shorter periods of time after gassing.

F. Bacteria in Non-Gassed Lungs

1. In Normal Lungs.—Cultures made from the trachea and bronchi of normal dogs showed plentiful growth. From the lung tissues in most cases there was no growth. Sections of normal lungs stained for bacteria showed a few bacteria on the bronchial mucosa but none in the alveolar structure.

2. In Cases of Experimentally Produced Pneumonia.—Pneumonia

TABLE VI

Results Showing Bacteria Present in Pneumonias Produced by the Inhalation or Injection of Irritating Substances Other than War Gases

Animal	Substance	Method	Death	Condition of lungs	Bacteria	
					Culture	Stain
Dog 5	N_2O_2	Inhalation	Killed, $MgSO_4$	Edema widespread. Exudate of leucocytes, fibrin, red cells	Diplococci	Occasional diplococcus. Few in edema fluid
Rabbit 16	$CuSO_4$ (1% solution)	Intratracheally 1 cc.	Killed	Normal	None	
Rabbit 15	$CuSO_4$ (1% solution)	Intratracheally 1 cc.	Died 11 days	Purulent pleurisy consolidation all lobes	Many colonies	Diplococci
Dog 35-82	$CuSO_4$ (2% solution)	Intratracheally 4 cc.	Died 2 days	Necrosis of trachea, mediastinum and pericardium. Congestion of lungs	Many colonies	Diplococci
Dog 35-83	$CuSO_4$ (2% solution)	Intratracheally 6 cc.	Died 2 days	Edema around trachea and mediastinum and pericardium, necrosis of tracheal mucosa and hemorrhage, edema, consolidation of lungs	Several colonies	Staphylococci, rods
Dog 35-92	$CuSO_4$ (1.5% solution)	Intratracheally 6 cc.	Died 1 day	Edema	No growth. Few colonies from bronchi	Diplococci
Dog 35-93	$CuSO_4$ (1.5% solution)	Intratracheally 5 cc.	Killed 2 days	Congestion, hemorrhage consolidation of lungs	Few colonies	Very minute rods
Dog 35-86	Ethylbromacetate	Intravenously	Died 2 days	Hemorrhage, atelectasis, consolidation of lungs	Few colonies	Diplococci

was induced in animals by the inhalation or injection of irritating substances, in order to compare the condition caused with that of gassed lungs. Table VI shows the results. Pneumonia was produced by the inhalation of nitric oxide fumes, by the intratracheal injection

TABLE VII

Results Showing Relation of Bacteria to Exudate in Dogs Dying with Spontaneously Developed Pneumonia

Animal	Death	Condition of lungs	Bacteria	
			Cultures	Stain
Dog 6	Killed, $MgSO_4$	Consolidation of lungs. Enormous hypertrophy of bronchial mucosa. Bronchial exudate, glands dilated. Alveolar walls thick	Colonies from all lobes	Rods and staphylococci
Dog 7	Died	Consolidation of lungs. Edema. Exudate of epithelial cells, mononuclears, few leucocytes		Staphylococci, few diplococci
Dog 9	Died	Consolidation of lungs. Exudate of leucocytes		Large numbers of bacteria, intracellular
Dog 10	Died	Consolidation all lobes. Exudate of leucocytes and mononuclears		Many bacteria, most of them intracellular
Dog 11	Died	Consolidation all lobes. Exudate of leucocytes, mononuclears and epithelial cells		Many bacteria, intracellular
Dog 12	Died	Consolidation all lobes. Exudate of leucocytes and epithelial cells		Some bacteria, mainly intracellular
Dog 13	Died	Consolidation all lobes. Edema. Exudate of leucocytes, mononuclears and epithelial cells		Many bacteria, intracellular

of copper sulfate solution, and by the intravenous injection of ethylbromacetate. None of these pneumonias were sterile.

3. Pneumonia Occurring Spontaneously in Dogs.—A number of dogs ng in the kennels were autopsied and found to have consolidation the lungs. These animals had not been gassed at any time. A few were killed with an injection into the heart of concentrated magnesium sulfate solution, when seen to be in bad condition.

The findings in this series are given in Table VII.

In a number of these animals the pneumonic exudate consisted largely of desquamated epithelial cells—the "desquamative pneumonia" of Buhl (13), Fraenkel (14) and Galdi (15), or the "chemical pneumonia" of other authors. That the anatomical picture, which Wood (2) claims is often produced by chemical agents without the aid of bacteria, can be produced by bacteria alone, is here clearly demonstrated. Furthermore, Galdi (15) has described similar pneumonias in which the infection occurred by the hematogenous route as a sequel to ulcerative endocarditis.

V. CONCLUSIONS

The question of a causal relation of the bacteria in gassed lungs to the pneumonia present cannot be regarded as decided. It may be said that:

1. The appearance of gassed lungs with pneumonia is very similar to the pneumonia of known bacterial origin.

2. In a few cases the type of pneumonia found coincides with the reported cases of so-called "chemical pneumonia," which is characterized by a preponderance of epithelial cells in the exudate.

3. Gassed lungs are not sterile but show highly varying numbers of bacteria.

4. The bacteria are not intracellular and are not present in large numbers in the majority of cases.

The arguments for and against a causal relationship between the bacteria and the pneumonia may be summed up as follows:

1. Against a Causal Relationship

a. The early appearance of pneumonia after gassing.

b. The occurrence of pneumonia with very small numbers of bacteria present.

c. The fact that very few bacteria are engulfed by leucocytes in gassed lungs, whereas large numbers are present in the non-gassed pneumonias and are conspicuously intracellular.

2. In Favor of a Causal Relationship

a. The presence of bacteria in any numbers.

b. The picture of broncho-pneumonia presented is similar to broncho-pneumonia of known bacterial origin.

c. Pneumonias characterized by large numbers of epithelial cells in the exudate (so-called "chemical pneumonia") occur in animals that were never gassed or subjected to other irritating substances in any way.

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EXPLANATION OF PLATES

PLATE 4

FIG. 1. Desquamative pneumonia from lung of dog gassed with phosgene. Symptoms disappeared in 2 days. Killed 1 week later.

FIG. 2. Patch of desquamative pneumonia in a dog that recovered from mustard gassing and was killed 9 months after recovery.

PLATE 5

FIG. 3. Desquamative pneumonia in a dog that was never gassed or exposed to irritating substances of any kind. Killed because it was thought he had worms. Positive culture and bacterial stain from lungs.

FIG. 4. Nitric oxide pneumonia—2 days—leucocytic type.

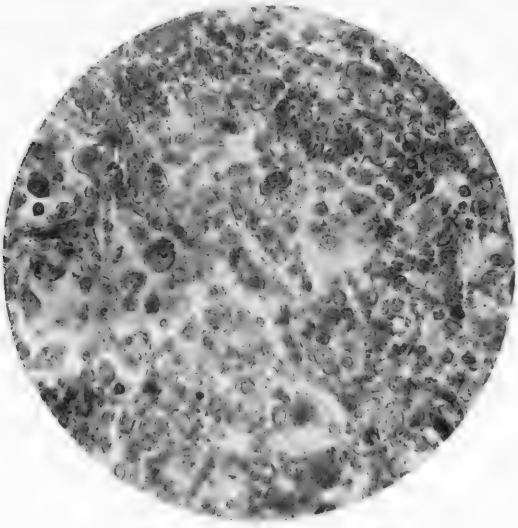


FIG. 1

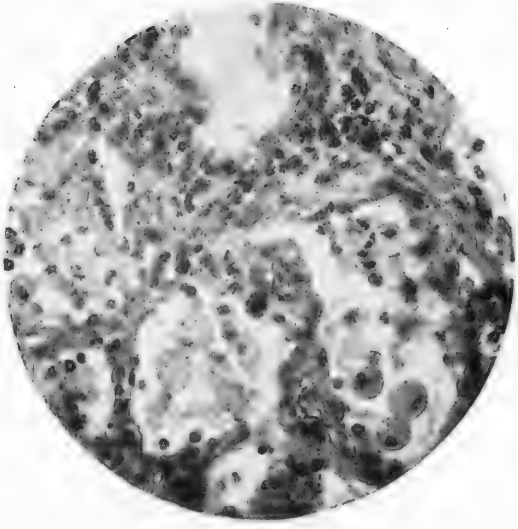


FIG. 2

(Koontz and Allen: Relation of bacteria to "chemical pneumonia")

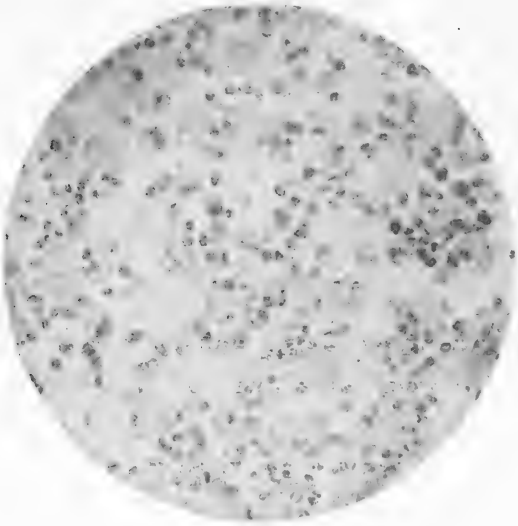


FIG. 3

FIG. 4

(Koontz and Allen: Relation of bacteria to "chemical pneumonia")

UNIV. OF MICH.

STUDIES ON SOUTH AMERICAN YELLOW FEVER

III. Transmission of the Virus to Brazilian Monkeys
Preliminary Observations

By NELSON C. DAVIS, M.D., and RAYMOND C. SHANNON

(From the International Health Division of the Rockefeller Foundation, New York, and the Yellow Fever Laboratory, Bahia, Brazil)

(Received for publication, April 23, 1929)

It is our intention to state briefly some of the results obtained in attempts to transmit yellow fever virus to the species of Brazilian monkey popularly known as "prego." This species was used by Noguchi, identified as *Cebus macrocephalus*, and found by him to be susceptible to *Leptospira* infections; the lesions produced closely simulated those of human yellow fever.

The temperature of all animals inoculated with the virus was taken morning and evening, and in a few instances it rose above 104°F. In several instances the fever appeared early and continued for varying intervals, usually with a tendency to intermissions. It is not improbable that the prolonged fevers may have been due to some other condition than to the inoculation with the virus.

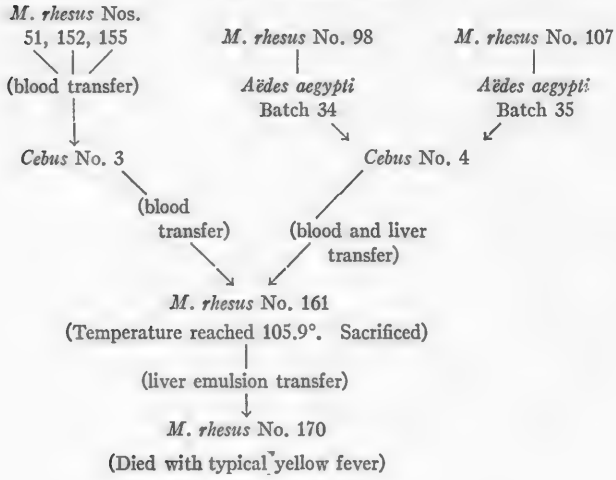
Some animals reacted mildly although the material inoculated was highly virulent for *Macacus rhesus*. Monkeys 6, 9, 12 and 24 fall in this category. In instances in which the inoculum was injected into two animals the effects might vary considerably (Nos. 1 and 2, and 19 and 20).

The virus has been passed from *rhesus* monkeys to *Cebus* and back to *rhesus* by the inoculation of blood and tissues.

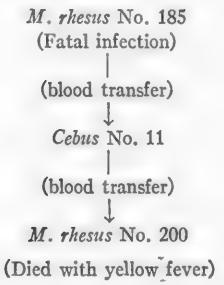
On Nov. 16, 1928, *rhesus* No. 161 was given 5 cc. of blood from *Cebus* No. 3 (taken on second day following inoculation); on Nov. 18, 5 cc. of blood from *Cebus* No. 4 (24 hours after mosquito feeding) was inoculated into the same *rhesus* and on the 19th a further transfer of 7 cc. blood-liver mixture was made from *Cebus* No. 4. On Nov. 20 the temperature of No. 161 rose to 105.2°F. and on the 21st it reached 105.9°. The animal was then sacrificed. Liver emulsion from No. 161 was inoculated into *rhesus* No. 170; the latter died and showed typical

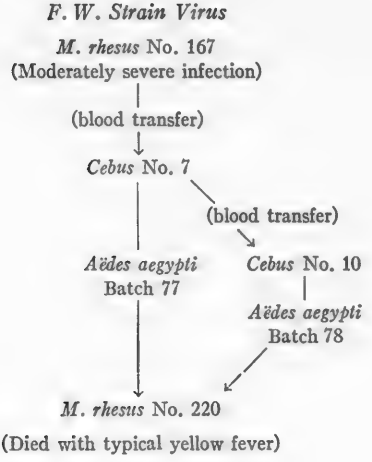
Schematic Representation of Transfers Mentioned in the Text

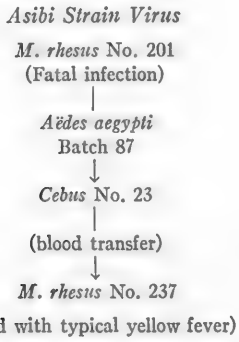
B. B. Strain Virus



Asibi Strain Virus




F. W. Strain Virus
M. rhesus No. 167
(Moderately severe infection)
(blood transfer)
Cebus No. 7
(blood transfer)
Aëdes aegypti
Batch 77
Cebus No. 10
Aëdes aegypti
Batch 78
M. rhesus No. 220
(Died with typical yellow fever)


Asibi Strain Virus
M. rhesus No. 201
(Fatal infection)
Aëdes aegypti
Batch 87
Cebus No. 23
(blood transfer)
M. rhesus No. 237
(Died with typical yellow fever)

gross and microscopic lesions of yellow fever. On Dec. 10, 1928, *rhesus* No. 200 was injected with blood from *Cebus* No. 11 (taken on the second day after inoculation); again on Dec. 12 No. 200 received blood from the same *Cebus* (taken on the fifth day). No definite fever was detected but on Dec. 14 the temperature fell to 96.4° and the monkey died during the night. The gross picture was typical of yellow fever; microscopically, both liver and kidney showed pronounced necrosis.

The virus has been passed to *Cebus* monkeys by blood inoculation and back to *rhesus* by mosquitoes (*Aëdes aegypti*).

On Dec. 4 *Cebus* No. 7 was inoculated with blood from *rhesus* No. 167, F. W. strain. On the 6th mosquito Batch 77 was allowed to feed and blood was transferred to *Cebus* No. 10. Mosquito Batch 78 was allowed to feed on the latter animal twice (Dec. 7 and 8). On Jan. 2 Batches 77 and 78 fed on normal *rhesus* No. 220. On Jan. 5 this animal registered a temperature of 105.2°F.; fever lasted 3 days and the monkey was found dead Jan. 9. The autopsy lesions were typical of yellow fever. Mosquito Batch 84, fed on *Cebus* No. 12 (B.B. strain), led to a febrile reaction in *rhesus* No. 222, but the animal survived.

The virus has been passed to *Cebus* monkeys by mosquitoes and back to *rhesus* by blood inoculation.

On Jan. 8, 1929, mosquito Batch 87 (infected from *rhesus* No. 201, Asibi strain) was allowed to feed on *Cebus* No. 23. On Jan. 10 this animal had a temperature of 104.2°F., and 1 cc. of blood was transferred to *rhesus* No. 237 by intraperitoneal inoculation. On Jan. 14 No. 237 showed a temperature of 105.6°; on the morning of the 16th the temperature was 96.0°F. and the animal died during the forenoon. Autopsy lesions were typical of yellow fever.

None of the *Cebus* of this series which died or were killed showed definite lesions of yellow fever, either in the gross or microscopically. The work is being continued with the species.

SUMMARY

Yellow fever virus from *M. rhesus* has been inoculated into a South American monkey (*Cebus macrocephalus*) by blood injection and by bites of infected mosquitoes. The *Cebus* does not develop the clinical or pathological signs of yellow fever. Nevertheless, the virus persists in the *Cebus* for a time as shown by the typical symptoms and lesions which develop when the susceptible *M. rhesus* is inoculated from a *Cebus* by direct transfer of blood or by mosquito (*A. aegypti*) transmission.

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IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND

IV. The Effect of Repeated Injections of Epinephrine on the Hemolysin Formation in Suprarenalectomized Rats

By DAVID PERLA, M.D., and J. MARMORSTON-GOTTESMAN, M.D.

(From the Division of Laboratories, Montefiore Hospital, New York)

(Received for publication, May 4, 1929)

In previous studies (1)* it was found that epinephrine subcutaneously injected into normal rats in amounts of 0.2 mg. per kilo twice daily during 3 days prior and 4 days subsequent to the injection of 1 cc. of a 10 per cent suspension of sheep cells depressed the antibody forming capacity of normal rats to a marked degree. The average titer was 1/550. In an effort to determine whether the depression of the antibody forming capacity of suprarenalectomized rats (2) is due to a lack of epinephrine the following experiments have been undertaken.

The experiments were divided into two groups. The first group included 30 rats. Of these, 18 were suprarenalectomized, 9 were normal and 3 were rats in which the perisuprarenal fat was torn around both glands. Twelve suprarenalectomized rats were injected subcutaneously with epinephrine in amounts of 0.2 mg. per kilo twice daily from the day of operation during 3 days prior and 4 days subsequent to the injection of 1 cc. of a 10 per cent suspension of sheep cells. Three suprarenalectomized rats received no injections, three suprarenalectomized rats received 0.5 cc. of a 0.9 per cent salt solution twice daily. Of the normal controls, three normal rats received no fluid injections, three received the same amounts of epinephrine as the suprarenalectomized group, three normal rats received physiological salt solution. Three rats in which the perisuprarenal fat was traumatized also received injections of physiological salt solution in the same amounts as the above groups.

In all the experiments recorded, Mulford's "Adrin" (1/1000) was used. In the first group of experiments dilutions were made with a 0.9 per cent salt solution

* The literature on the effect of epinephrine on antibody formation in normal rats has been reviewed in a previous communication (2).

TABLE I

Hemolysin Formation After Epinephrine-salt Solution Injections in Suprarenalectomized Rats

Rat No.	5th day	8th day	11th day	Weight at opera.	Weight at last titer
				gm.	*gm.*
370	1600	1000	400	195	187
371	2000	2000		180	180
372	8000			190	180
373	2000	1000	400	180	170
374	600	100		220	192
375	6000	2000		200	197
431	600	160		190	180
432	1000			190	190
433	2000			197	155
434	3000	1000	200	175	175
435	80	200	300	225	180
436	1000	300	600	250	220
Av. titer....	2323	862	380		
Suprarenalectomized Controls					
455	400	40	100	160	145
450	200			235	220
453	1000	4000		175	145
Suprarenalectomized Saline Controls					
438	160	100	60	175	150
439	12000	1000	200	195	190
440	3000	1000	300	150	120
Normal Controls					
427	1600			275	285
428	6000	2000		280	292
501	4000	3000	200	140	145
Normals + Adrin					
446	800	100	100	150	155
447	80	30	60	145	150
448	80	1000		135	140
*Normals + Saline**					
462	8000	2000	800	215	220
463	16000	12000	6000	205	215
Traumatized + Saline					
441	3000	1000	400	180	175
442	3000	1000	300	155	160
443	16000	1000	400	185	175

* Physiological salt solution injected in amounts of 0.5 cc. subcutaneously twice daily during 3 days prior and 4 days subsequent to injection of sheep cells.

and all injections were administered subcutaneously. The amounts used for each rat were brought up to a total volume of 1 cc. with freshly boiled physiological salt solution and 0.5 cc. was injected twice daily. In all instances a 24 hour period was allowed to intervene before and after the injection of sheep cells, during which time no epinephrine was administered. The method of titration has been described in a previous communication (2).

TABLE II

Hemolysin Formation After Epinephrine-glucose Injections in Suprarenalectomized Rats

Rat No.	Titers			Weight at opera.	Weight at last titer
	5th day	8th day	11th day		
				gm.	*gm.*
833	1600	160	80	194	178
834	1600	250	100	154	154
835	3000	1000	600	166	166
836	3000	300	160	194	195
837	3000	600	60	230	197
838	4000	700	160	208	183
839	400	400	300	200	181
840	1600	—	—	203	190
841	3000	600	200	185	172
Av. titer....	2355	501	207		
Suprarenalectomized Rats Untreated					
843	400	100	60	143	119
844	3000	800	600	200	178
845	1000	400	200	134	120
Normal Rats Untreated					
846	3000	200	100	185	191
847	5000	400	200	130	135

The results of these experiments are tabulated in Table I. It is seen that injections of epinephrine diluted with salt solution are followed by a definite rise in antibody formation in suprarenalectomized rats. The average hemolysin titer of the rats in this group was 1/2383 whereas that of the suprarenalectomized rats not treated with epinephrine was 1/858. About 50 per cent of the rats injected with epinephrine yielded titers above 1/2000.

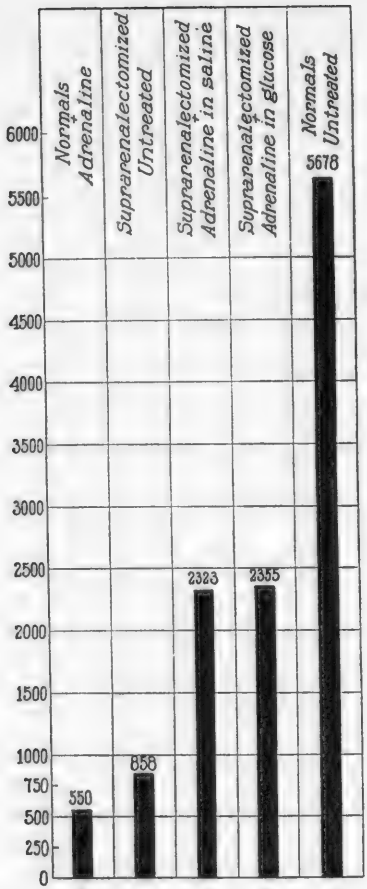

FIG. 1

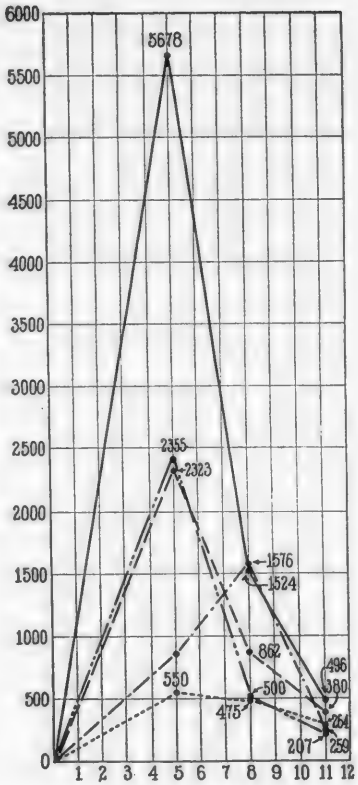

FIG. 2

FIG. 1. The average hemolysin titers of normal and suprarenalectomized rats repeatedly injected with epinephrine diluted with physiological salt solution and with isotonic glucose solution.

FIG. 2. The curves of the average hemolysin titers of normal and suprarenalectomized rats repeatedly injected with epinephrine diluted with physiological salt solution and with isotonic glucose solution.

——— Normal rats.

--------- Normal rats injected with epinephrine.

— — — Suprarenalectomized rats injected with epinephrine diluted with salt solution.

— - — - Suprarenalectomized rats injected with epinephrine diluted with glucose solution.

——— Suprarenalectomized rats uninjected.

It was observed, however, that the suprarenalectomized rats injected with 0.5 cc. of a 0.9 per cent salt solution also showed a marked rise in antibody formation.

In an effort to eliminate any possible effect of the physiological salt solution used in diluting the epinephrine a second group of experiments was done in which the epinephrine was diluted with isotonic glucose solution (see Table II). Ten suprarenalectomized rats were injected with 0.2 mg. per kilo of epinephrine (diluted with glucose solution) twice daily during 3 days prior and 4 days subsequent to the injection of the antigen. Three suprarenalectomized rats received no injection and three normal rats received no injection. The average titer of the glucose-epinephrine injected rats was 1/2355 or about the same as that of the first group of experiments. This titer is three times as great as that of the untreated suprarenalectomized rats, but still considerably lower than the average titer in normal rats.

DISCUSSION

It may be concluded from these experiments that the injection of epinephrine raises the hemolysin titer in suprarenalectomized rats and that the effect is not entirely dependent on the salt solution with which the epinephrine is diluted. The effect of epinephrine diluted with salt solution is not a summation of both epinephrine and salt solution. We do not believe that the loss in epinephrine resulting from suprarenalectomy is alone responsible for the resulting depression in antibody formation. There is some evidence however from these experiments that it plays a part in the antibody forming mechanism.

There is a striking difference in the effect of epinephrine injections on the hemolysin formation of normal and of suprarenalectomized rats. In normal rats, the hemolysin titer was markedly depressed, in suprarenalectomized rats it was raised above the level of untreated suprarenalectomized rats. An excess of epinephrine supplied to rats possessing their physiological store of epinephrine depresses the mechanism of antibody formation. Rats, however, deprived of a physiological source of epinephrine and injected with the same amounts as in the previous group are stimulated to increased antibody formation.

CONCLUSIONS

Epinephrine diluted with physiological salt solution or with isotonic glucose solutions and injected in amounts of 0.2 mg. per kilo twice daily into suprarenalectomized rats raises their antibody forming capacity. This result is not entirely dependent on the effect of the diluting fluid.

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IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND

V. The Effect of Repeated Injections of Solutions Containing Sodium Salts and Glucose on the Hemolysin Formation of Normal and Suprarenalectomized Rats

By J. MARMORSTON-GOTTESMAN, M.D., and DAVID PERLA, M.D.

(*From the Division of Laboratories, Montefiore Hospital, New York*)

(Received for publication, May 4, 1929)

In previous studies (1) on hemolysin formation in normal adult albino rats, it was found that 20 normal rats yielded an average titer of 1/7000, while in 23 additional normal rats the average titer was 1/4323. The average titer of the 43 rats collected in both series was 1/5656, somewhat lower than had previously been estimated with half the number of animals. Seventy per cent of these rats gave titers above 1/2000. An occasional very low reading was noted. From an analysis of the factors responsible for this variation it was observed that the females yielded somewhat higher hemolysin titers than the males (females 1/6850, males 1/4620). Older rats gave a higher antibody titer than young adult rats. Rats of greater weight but of the same age and sex yielded higher titers.

All the rats used in these experiments were bred in the laboratory under the same environmental conditions and were fed on a constant adequate diet. The variations observed in these animals in spite of these constant factors emphasize the necessity of maintaining the factors of age and weight constant and of utilizing large numbers of animals in studies on antibody formation. In our later studies only rats about 3 months of age were used and the results were more uniform.

It was found in previous experiments (2) that suprarenalectomy is followed by a depression in antibody forming capacity most marked during the first week following the operation. Further studies on hemolysin formation in this early period confirmed this observation. Of a series of 23 suprarenalectomized rats injected on the third day

following the operation with 1 cc. of a 10 per cent suspension of sheep cells and tested 5 days later, the average hemolysin titer was 1/858 as compared with 1/5650 of the normal rats. About 70 per cent of suprarenalectomized rats gave titers below 1/2000. Two rats gave high titers corresponding to normal figures. This may be explained by the well known fact that a certain small percentage of suprarenalectomized rats react as normal rats, due to large amounts of accessory cortical tissue (3). Of the suprarenalectomized rats, as of the normal rats, the females gave slightly higher titers than the males.

In the course of experiments on the effect of injections of epinephrine on the hemolysin formation in suprarenalectomized rats (4) it was noted that repeated injections of epinephrine in amounts of 0.2 mg. per kilo raised the hemolysin titer. Small amounts of physiological salt solution raised the titer to about the same level as the epinephrine injected group. This observation suggested the experiments reported in this communication. Studies on hemolysin formation were made in suprarenalectomized rats injected repeatedly with large quantities of a 0.9 per cent salt solution, isotonic sodium acetate, isotonic glucose solution and hypertonic salt solution in order to determine the effect of these substances on antibody production.

Methods

The method of operation, the post-operative care of the animals and the exact procedure of obtaining the blood and titrating the serum has been described in detail in previous communications (5) (2).

In all the experiments reported in this paper, 1 cc. of a 10 per cent suspension of sheep cells was injected intraperitoneally and titers were studied on the 5th, 8th and 11th day following the injection. When suprarenalectomized rats were studied the antigen was injected 48 to 72 hours after operation and the first titer was read on the 7th or 8th day after suprarenalectomy.

The Effect of Injections of Physiological Salt Solution on Hemolysin Formation in Normal and Suprarenalectomized Rats

Four groups of rats were tested. One group of 26 normal rats received two daily injections of 0.5 cc. of physiological salt solution during 3 days prior and 4 days subsequent to the injection of antigen. The average titer in these rats was 1/5516 or about the same as untreated normal rats. A second group of six normal rats

received 5 cc. of physiological salt solution intraperitoneally, once daily during 3 days prior and 4 days subsequent to the injection of sheep cells. The average titer was 1/4133, slightly lower than the titer of normal control rats.*

A third group of thirteen suprarenalectomized rats received 0.5 cc. of physiological salt solution subcutaneously twice daily. The average titer in this group was 1/2630 as compared with 1/858 of the untreated suprarenalectomized rats.

A fourth group of ten suprarenalectomized rats received 5 cc. of physiological salt solution intraperitoneally once daily from the day of operation to the day of the last reading. The average titer in this group was 1/3580, strikingly higher than the titer of untreated suprarenalectomized rats. The general condition of suprarenalectomized rats that had received salt solution injections was excellent. They ate well, exercised vigorously, kept their fur white and sleek and appeared stronger than the uninjected rats. These results indicate a marked beneficial effect from repeated injections of 0.9 per cent salt solution on the general well being and antibody forming capacity of suprarenalectomized rats.

Effect of Injections of Isotonic Sodium Acetate Solution on the Hemolysin Formation of Suprarenalectomized Rats

In an effort to determine the factors responsible for the rise in hemolysin titer in suprarenalectomized rats injected with physiological salt solution, other solutions were used. Isotonic sodium acetate solution was injected into ten suprarenalectomized rats in the same quantities by volume and over the same period of time as those injected with physiological salt solution. The average titer was 1/4260 or about five times as high as the untreated suprarenalectomized rats (1/858). It is apparent therefore that the chlorine ion is not essential

* Belak and Csereszynes (6) found some stimulating effect of physiological salt solution on the agglutinin formation following the injection of paratyphoid B bacilli in normal rabbits. They injected 0.5 cc. of the solution daily for 14 days after the injection of the antigen. Ringer's solution and isotonic calcium chloride had the same effect. They do not believe the sodium ion was responsible for the rise. Their data were collected on four rabbits. We were unable to demonstrate analogous findings in the case of hemolysin formation in normal rats.

in raising the antibody titer in suprarenalectomized rats. Normal control rats injected with isotonic sodium acetate solution in amounts of 5 cc. per day during 3 days prior and 4 days subsequent to the injection of sheep cells yielded titers slightly lower than untreated normal rats.

Effect of Repeated Injections of Isotonic Glucose Solution on the Hemolysin Titer of Suprarenalectomized Rats

To determine whether the effect of salt solution on the antibody formation in these rats was due to its diuretic effect, 5 cc. of isotonic glucose solution was injected once daily intraperitoneally into eight suprarenalectomized rats during the entire period from the day of operation to the day of the last reading of the hemolysin titer. The average titer of this group was 1/887, or about the same as that of untreated suprarenalectomized rats. In spite of its diuretic action, isotonic glucose did not raise the antibody formation of suprarenalectomized rats. In a group of normal control rats which were injected with isotonic glucose solution there was almost no change in the hemolysin titers from that of uninjected normal rats. The average titer in this group was 1/4500.

The Effect of Concentrated Solution of Sodium Chloride on the Hemolysin Formation of Suprarenalectomized Rats

An effort was made to determine whether sodium chloride alone had the same effect as large volumes of isotonic salt solution. 0.5 cc. of a 10 per cent solution of sodium chloride was injected into eight suprarenalectomized rats once daily from the day of operation during 3 days prior and 4 days subsequent to the injection of sheep cells. The hypertonic solution was extremely irritating. It was observed that the concentrated salt solution had only a slight effect in raising the antibody formation in suprarenalectomized rats. The average titer in this group was 1/1660.

DISCUSSION

It is apparent from these data that injections of isotonic solutions of sodium salts raise the titer of suprarenalectomized rats. This rise in hemolysin titer is not due entirely to the diuretic effect of the large

volume of salt solution injected, since the same volume of glucose solution, an excellent diuretic, has no such effect. It was found that sodium chloride administered in a small volume of fluid but in the same amount by weight as in the isotonic solutions has only a slight effect in raising the titer in suprarenalectomized rats. It would seem from these observations that both the fluid and the sodium ion are necessary to bring about a restoration of the antibody forming capacity of suprarenalectomized rats to normal.

Our results are in accord with the findings of Marine and Baumann (7). They found that the administration of Ringer's solution and isotonic solutions of sodium chloride and sodium acetate increased the duration of life of suprarenalectomized cats about three times. More concentrated solutions of sodium chloride definitely shorten life. Isotonic solutions of glucose and glycerol had only a very slight life-prolonging effect. They concluded from their studies that diuresis is one of the important factors in determining the duration of life. When the loss of water by diuresis is compensated for by an additional intake, life is prolonged and if not, life is shortened. There is some indication, they believe, that the loss of sodium is more specific than can be accounted for as a result of a possible acidosis.

It is well known that the sodium ion plays an important rôle in the water exchange in the tissues. Suprarenalectomy results in a profound disturbance of the water balance in the body. Following the removal of the glands, there is a loss of fluid through the intestines, a diminution of sodium concentration in the blood (Baumann and Kurland (8)) and an impairment of kidney function. These factors result in tissue dehydration and a disturbance of the normal cellular physiological activity. Those tissue elements concerned with antibody production are less able to respond to the stimulation of foreign substances with the production of immune bodies in suprarenalectomized rats than in normal rats. There may, however, be no actual loss of immune body production but a diminished release of these antibodies into the blood stream. The restoration of the water balance by the promotion of diuresis with a simultaneous, increased intake of fluid in a form that can be retained in the tissues helps to restore the mechanism of antibody production to the normal. The rôle of the suprarenal gland in antibody formation appears to be intimately connected with the maintenance of water balance.

The relation of a disturbance in the water balance of the tissues to

TABLE I

Fluid injected	Route of injection	Amount per injection	No. of rats	Titers											
				5th day				8th day				11th day			
				Maximum	Minimum	Mean	Average	Maximum	Minimum	Mean	Average	Maximum	Minimum	Mean	Average
Suprarenalectomized Rats															
none	—	—	21	3000*	0	800	858	4000	60	1200	1576	1000	0	200	259
isotonic sod. chlor.	subcut.	0.5 cc. twice daily	13	12000	160	2000	2630	1600	100	600	709	400	60	200	204
" " "	intrap.	5 " once "	10	16000	1000	3000	3580	600	40	350	354	200	40	100	110
isotonic glucose	"	5 " " "	8	2000	400	800	887	1000	100	600	600	300	40	100	142
isotonic sod. acet.	"	5 " " "	10	8000	1600	4000	4260	2000	300	1000	990	800	80	200	280
hypertonic sod. chlor.	"	5 " " "	7	2000	1000	2000	1660	1600	600	1200	1240	800	150	300	400
Normal Rats															
none	—	—	43	32000	100	4000	5678	16000	10	1600	1524	2000	10	400	496
isotonic sod. chlor.	subcut.	0.5 cc. twice daily	26	32000	160	4000	5516	12000	80	1600	2000	6000	0	300	582
" " "	intrap.	5 " once "	6	8000	1800	4000	4133	3000	1000	1000	1500	600	300	300	380
isotonic glucose	"	5 " " "	6	8000	3000	4000	4500	3000	800	1500	1800	800	400	600	580

*A single high reading of 16000 was omitted from average.

antibody formation has hitherto not been noted by previous investigators to the authors' knowledge. This observation is of importance

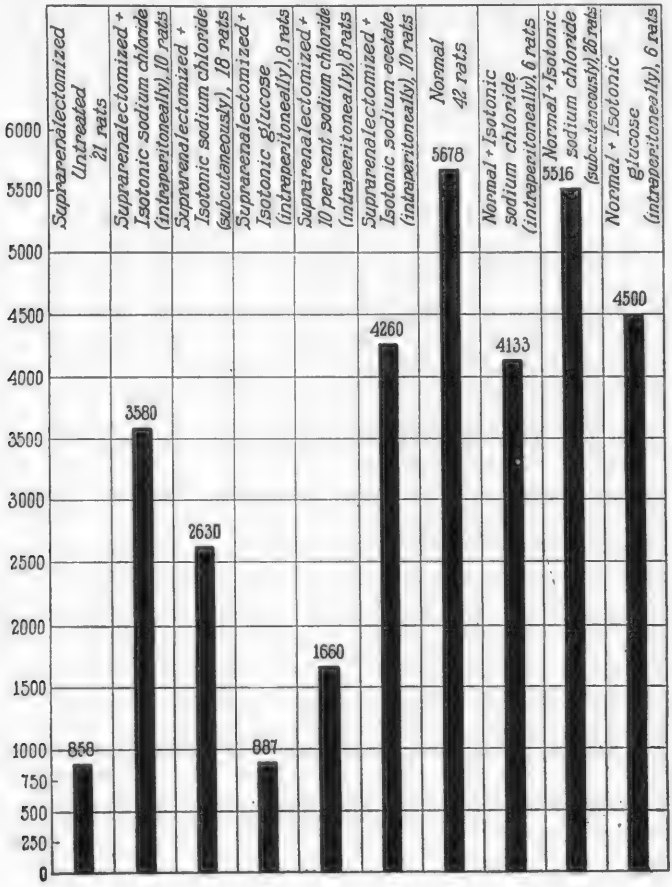

FIG. 1. The effect of solutions containing sodium salt and glucose on the hemolysin formation of normal and suprarenalectomized rats. The columns represent the average hemolysin titer for each group.

in the interpretation of certain phenomena in acute infections. It is a matter of empiricism that "forcing fluids" is beneficial in the treat-

ment of severe infections and in extensive skin burns. It is known that in many acute infections, and especially in burns, the suprarenal

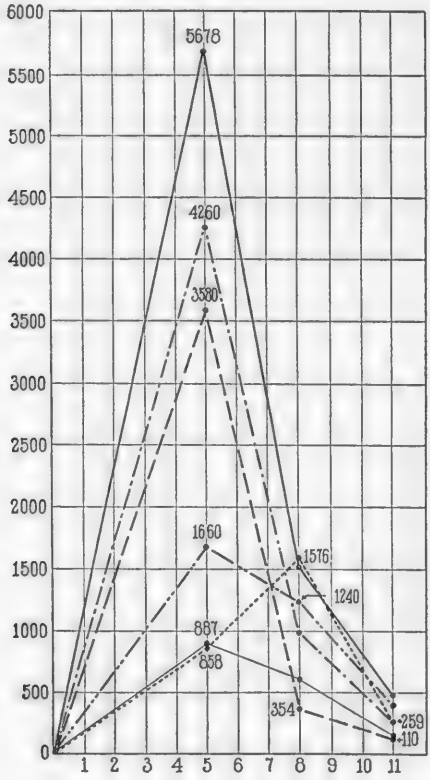

FIG. 2. The curves of the average hemolysin titers of normal and suprarenalectomized rats repeatedly injected with solutions containing sodium salts and glucose.

——— Normal rats.
- - - - - - - - Suprarenalectomized rats untreated.
— — — Suprarenalectomized rats treated with physiological salt solution.
—— - —— Suprarenalectomized rats treated with isotonic sodium acetate solution.
——— Suprarenalectomized rats treated with isotonic glucose solution.
— - - - Suprarenalectomized rats treated with 10 per cent sodium chloride solution.

glands are severely injured. This injury in turn may result in a disturbance of the tissue water balance and in the antibody forming capacity of the body. This disturbance may be corrected to some extent by parenteral and oral administration of large quantities of physiological salt solution.

In their recent studies on experimentally produced peritonitis in dogs, Orr and Haden (9) injected 1 per cent solutions of sodium chloride and found that the animals lived three times as long as those not given the salt solution. This may be due to the effect of the salt solution on the mechanism of water metabolism disturbed as a result of the suprarenal injury which accompanies a general peritonitis.

SUMMARY

Table I and Figs. 1 and 2 summarize the experiments done on 150 rats. Suprarenalectomy in rats is followed by a striking depression in antibody formation most marked during the first week following the operation. Repeated injections of large amounts of isotonic sodium chloride or sodium acetate solutions restore the antibody titer to normal. The effect of these solutions is not due to diuresis alone, since isotonic glucose solution does not affect the titer, nor to the sodium alone, since hypertonic solutions of sodium chloride in small volume have little influence on the titer. Both the sodium ion and the water volume are necessary in the restoration of the titer.

CONCLUSION

(1) Suprarenalectomy is followed by a disturbance in the antibody forming mechanism of the body. (2) The suprarenal gland plays a rôle in the water metabolism of the tissues. (3) Restoration of the water exchange in the tissues to a normal level is sufficient to raise the antibody forming capacity to normal.

We are indebted to Dr. David Marine for his helpful suggestions in the course of this work.

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THE PRODUCTION AND TITRATION OF POTENT HORSE ANTIPNEUMOTOXIN

By JULIA T. PARKER and MARY Van S. McCOY

(*From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York*)

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In a previous communication[1] it was shown that certain anaerobically produced autolysates of pneumococci contain a poison which produces marked lung lesions when injected intratracheally into small guinea pigs. In a later paper,[2] this poison was shown to be neutralized by the serum of rabbits immunized thereto. These rabbit serums were of relatively low potency, containing approximately 50 neutralizing units per cubic centimeter (0.1 cc. of serum neutralized 5 lethal doses of the autolysate). In the present paper, we wish to report on the production of high titre anti-autolysate serums in the horse[3] and give in detail the method used for their titration.

For the sake of clearness, in this and subsequent papers, the lung-toxic poison will be called "pneumotoxin" and the neutralizing serums for this poison, "antipneumotoxin." We have as yet no absolute proof that the lung-toxic poison is antitoxinogenic, but apparently it is, as it fulfils most of the accepted characteristics of this class of poisons.

EXPERIMENTAL

For the last 8 months, two horses,—Nos. 725 and 726,—have been undergoing treatment with increasing doses of sterile filtrates of the pneumotoxin from Types I, II and III. The first injections of these filtrates were in doses of 0.5 cc. to 1 cc. and produced a marked rise in temperature. For the last 3 months, each of these horses has been

[1] Parker, J. T., and Pappenheimer, A. M., *J. Exp. Med.*, 1928, **48**, 695.

[2] Parker, J. T., *J. Exp. Med.*, 1929, **49**, 695.

[3] These horses were immunized to the autolysate filtrates at the Eli Lilly Company in Indianapolis, under the supervision of Mr. W. A. Jamieson.

receiving 400 cc. of the toxic autolysates every 3 or 4 days, without obvious reactions of any kind. Horse 787 has been receiving increasing doses of the pneumotoxin from pneumococcus Type I alone. Horse 382 had been receiving a variety of pneumococcus products, consisting of vaccines, broth cultures of the live pneumococcus, etc., since December 1925, but in November 1928, it was started on the pneumotoxin from Types I, II and III. Horse 685 had been on treatment with pneumococcus vaccine alone since December 1927, but in January 1929 these were discontinued and it was started on inoculations of pneumotoxin from pneumococcus Types I, II and III.

Method of Titration of the Serums for Neutralizing Antibodies to the Lung-toxic Poison

The tests for the presence of neutralizing antibodies were carried out as follows:—Dilutions of the serums in broth were first prepared. 0.1 cc. of the various serum dilutions, or broth instead of the serum dilutions, were pipetted into separate precipitin tubes which were kept in ice water. To each tube 0.9 cc. of chilled pneumotoxin was added and the contents immediately mixed. Heavy vaseline seals were then added to all the tubes, care being taken to avoid the formation of bubbles between the surface of the mixture and the vaseline. The tubes were then left at room temperature in the dark for 2 hours. After this, they were again placed in ice water, the vaseline seals removed and the contents of each tube pipetted into a chilled Wassermann tube. One or two guinea pigs were then injected intratracheally with 0.2 cc. of each preparation. To avoid the danger of oxidation by exposure to air, the vaseline seals were not removed until immediately before the inoculation of the guinea pigs. Guinea pigs weighing from 190 to 210 gm. were used in all the experiments.

Pneumotoxin from Types I, II or III were employed, autolysates from one type only being used in each experiment. These toxins were of such strength, that 0.2 cc. of mixtures of 0.9 cc. of toxin and 0.1 cc. of broth (0.18 cc. of toxin) invariably killed guinea pigs of 200 to 210 gm. in less than 24 hours with typical symptoms and autopsy findings.

One unit of toxin is the amount which, when injected intratracheally, will kill a guinea pig weighing 200 to 210 gm. in from 4 to 24 hours,

with typical symptoms and autopsy findings; while one unit of antitoxin represents the smallest amount of serum which is necessary to protect a guinea pig of the same weight against one unit of toxin when the mixture is injected intratracheally.

Results from These Neutralization Experiments

The strength of the horse serums in neutralizing antibodies for the lung-toxic poison appears to depend quite regularly on the amount of pneumococcus toxic autolysate the horses have received. The serums of Horses 725 and 726, the horses most highly immunized to the toxic autolysates, have shown a progressive and rapid increase in their content of neutralizing antibodies during the last 3 months (an increase of from 5000 to 40,000 units).

On the other hand, serums from several bleedings from Horse 787, which had recently been started on pneumococcus autolysate injections, and from Horses 382 and 685, which had been treated with a variety of pneumococcus products for several years and only recently with injections of toxic autolysates, all showed relatively small amounts of neutralizing antibodies. However, recent tests carried out with a concentrated preparation from the latest bleedings from Horses 382 and 685, show an increase in these antibodies. (See Table I.)

Effect of Normal Horse Serum, Antipneumococcus Horse Serum or Antipneumococcus Horse Serum Concentrated by the Felton Method[4] on the Lung-Toxic Autolysates

Neither normal horse serum nor unconcentrated antipneumococcus horse serum containing 500 protective units per cubic centimeter to Pneumococcus I had any detoxifying effect on the toxin when used in 1–10 dilution—0.1 cc. of serum added to 0.9 cc. of toxin—and set up under the same conditions as the other mixtures. The antipneumococcus serum concentrated by the Felton method and containing 1000 protective units per cubic centimeter to Pneumococcus I, detoxified the toxin when used in 1–20 dilution, but had no effect on the toxin when used in 1–50 dilution.

[4] We are indebted to Dr. William Park of the New York City Board of Health for these serums.

Experiment I. Table I

Serum Tested:

787. 4th bleeding.
725. 7th bleeding.
31222. Concentrated preparation from 6th bleeding of horses 725 and 726.
31221. Concentrated preparation from the 86th bleeding of Horse 382 and the 13th bleeding of Horse 685.

The in Vitro Neutralization of Lung-Toxic Autolysates with Autolysate Horse Serums

Pig No.	Weight	Serum tested	Final dilution of serum	Symptoms	Died or survived	Amt. consolidation in lungs	Calculated units of antitoxin per cc.
350	192	787	1–50	0	S.		
312	194	787	1–100	++	D <18 hrs.	+++	250
327	208	787	1–200	+++	D <18 hrs.	+++	
317	192	725	1–1000	0	S.		
308	206	725	1–2000	0	S.		20,000
346	190	725	1–4000	0	S.		
315	206	725	1–8000	+++	D <18 hrs.	++	
322	190	31222	1–2000	0	S.		
311	192	31222	1–4000	0	S.		20,000
314	204	31222	1–8000	++	D <48 hrs.	++	
321	206	31222	1–8000	+++	D <18 hrs.	+++	
325	190	31221	1–200	0	S.		
323	202	31221	1–400	0	S.		2,000
349	202	31221	1–1000	+++	D <40 hrs.	+++	
324	204	31221	1–2000	++	S.		
307	210	—	—	+++	D <18 hrs.	++	
309	208	—	—	+++	D <18 hrs.	++	
358	206	—	—	+++	D <18 hrs.	+++	

This table is self explanatory.

Table I shows the results of a typical experiment made to determine the strength of these neutralizing substances in the serum of immunized horses. There is one discrepancy in this experiment, in that Guinea pig 349, which received twice as much of Serum 31221 in its toxin-serum mixture as did Guinea pig 324, died; while the latter pig, although extremely sick for several days, survived. In tests carried out with the greater serum dilutions, discrepancies of this sort occa-

sionally occur. We believe that these discrepancies are due to the greater susceptibility to the toxin of a small percentage of pigs (approximately 10%), in which instances the lethal dose of toxin may be $\frac{1}{2}$ to $\frac{1}{4}$ the usual lethal dose. Therefore, for accurate results in the titration of a serum, it is necessary to use several pigs for each of the final dilutions of serum.

In a previous paper, it was shown that the pneumotoxin for Types I and II were antigenically similar. This experiment, Table I, brings out a point which we have recently confirmed several times, *viz.*, the pneumotoxin from Pn. Type III is also antigenically similar to toxins from Types I or II. (See Pig 350 in the table.)

Toxin used was a toxic autolysate from Pn. Type III. 0.9 cc. of the toxin was added to 0.1 cc. of the diluted serum, or to 0.1 cc. of broth. After standing for 2 hours at room temperature under vaseline seal, guinea pigs were injected intratracheally with 0.2 cc. of the mixtures.

CONCLUSIONS

1. The serum of horses immunized with increasing doses of certain anaerobically produced autolysates of pneumococci contain potent neutralizing antibodies for the pneumotoxin.

2. The method for the *in vitro* titration of these horse antipneumotoxic serums is given.

BIOMETRY OF CALCIUM, INORGANIC PHOSPHORUS, CHOLESTEROL, AND LECITHIN IN THE BLOOD OF RABBITS

IV. Effects of a Malignant Tumor

By ALVIN R. HARNES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

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The present paper is part of a study of the relations of certain blood constituents in animals as affected by various types of environment and disease (1, 2, 3). The experiments to be reported deal with the variations of calcium, inorganic phosphorus, lecithin and cholesterol in the blood of animals inoculated with a malignant neoplasm (4).

Material and Methods

The animals used were male rabbits from 6 to 8 months old at the beginning of the experiment. While under observation, the animals were caged separately, and kept in a well lighted (sunlight), well ventilated room with a southern exposure. The rooms were heated during the colder weather. The diet of the animals consisted of hay, oats, and cabbage.

Three groups of 10 animals each were inoculated in one testicle with 0.3 cc. of a tumor emulsion. Examinations of the blood were made at weekly intervals for a period of 2 months. During this time, some animals died; those that survived were then killed and autopsied.

The results for the control animals will be omitted as the general trend and variation of results are comparable to the group previously reported (2). Moreover, a comparison of the values obtained for animals that recovered with those for animals that died is more significant than a comparison made with normal values.

At the conclusion of the experiment, the 30 animals comprising this series were divided into 2 groups: (1) those animals in which death was due to the neoplasm, and (2) those recovering from the tumor inoculation. The results obtained from these 2 groups of animals are presented in the present paper. The results for the

12 animals in which death was due to the tumor (Group I) are presented in Table I, while the results of the 18 animals recovering from the tumor inoculation (Group II) are presented in Table II. Each table is divided into 7 dates of record, the first recorded examination, −14 days, are the results obtained 14 days preceding inoculation; second, the results obtained on the day of inoculation; third, the results +14 days after inoculation. The subsequent 4 determinations were made at 7 day intervals. The recorded values at "time of death" does not mean all animals died 42 days after inoculation, but summarizes the results obtained from these animals a day or two preceding expiration. The data presented in this paper are derived from experiments, the procedures of which were described in detail in a preceding paper (1). In all text-figures and tables the lipoid phosphorus is calculated and presented as lecithin.

The pertinent features of the experiment may be summarized as follows:

Group	No. of animals	No. of examinations	Date of inoculation	Observation period
A	10	99	Nov. 17, 1927	Oct. 27, 1927 to Jan. 12, 1928
B	10	99	Jan. 19, 1928	Jan. 5, 1928 to Mar. 22, 1928
C	10	76	Mar. 27, 1928	Mar. 15, 1928 to May 22, 1928

Results

The results of the observations made in this experiment are presented in the form of tabulated summaries, Tables I to III, which are supplemented by a series of graphs, Text-figs. 1 to 10. The mean values contained in Tables I and II have been smoothed by the formula $\frac{A + 2B + C}{4}$ and are presented in the graphs in terms of per cent variations from standard mean values, using for this purpose values obtained for animals living in the open laboratory throughout the year. These results were reported in a previous paper (2) and shall serve also as a basis of comparison for certain ratio values obtained in the present experiment.

TABLE I

Group I. Tumor Deaths

Days	Inorganic phosphorus			Calcium			Cholesterol			Lecithin		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
−14	5.59 ± 0.22	1.11	19.87	16.5 ±0.29	1.47	9.03	60.6 ±1.67	8.59	14.17	129 ±5.08	26.1	20.33
Inoculated	5.52 ± 0.25	1.29	23.37	15.0 ±0.17	0.87	5.80	60.0 ±1.61	8.26	13.77	113 ±4.30	22.1	19.56
+14	5.37 ± 0.12	0.63	11.75	14.8 ±0.18	0.95	6.42	63.7 ±1.27	6.51	10.22	118 ±5.47	28.1	23.81
+21	5.96 ± 0.22	1.11	18.62	14.7 ±0.13	0.67	4.56	64.4 ±1.75	8.97	13.93	108 ±5.82	29.9	27.68
+28	7.19 ± 0.91	4.65	64.67	14.4 ±0.23	1.17	8.13	69.0 ±2.09	10.72	15.54	123 ±3.29	16.9	13.74
+35	6.34 ± 0.38	1.95	30.76	15.4 ±0.37	1.88	12.21	80.5 ±6.09	31.28	38.86	110 ±1.42	7.3	6.64
Time of death	8.67 ± 1.10	5.67	65.39	13.9 ±0.26	1.33	9.57	91.5 ±6.42	32.98	36.04	120 ±3.82	19.6	16.33

TABLE II

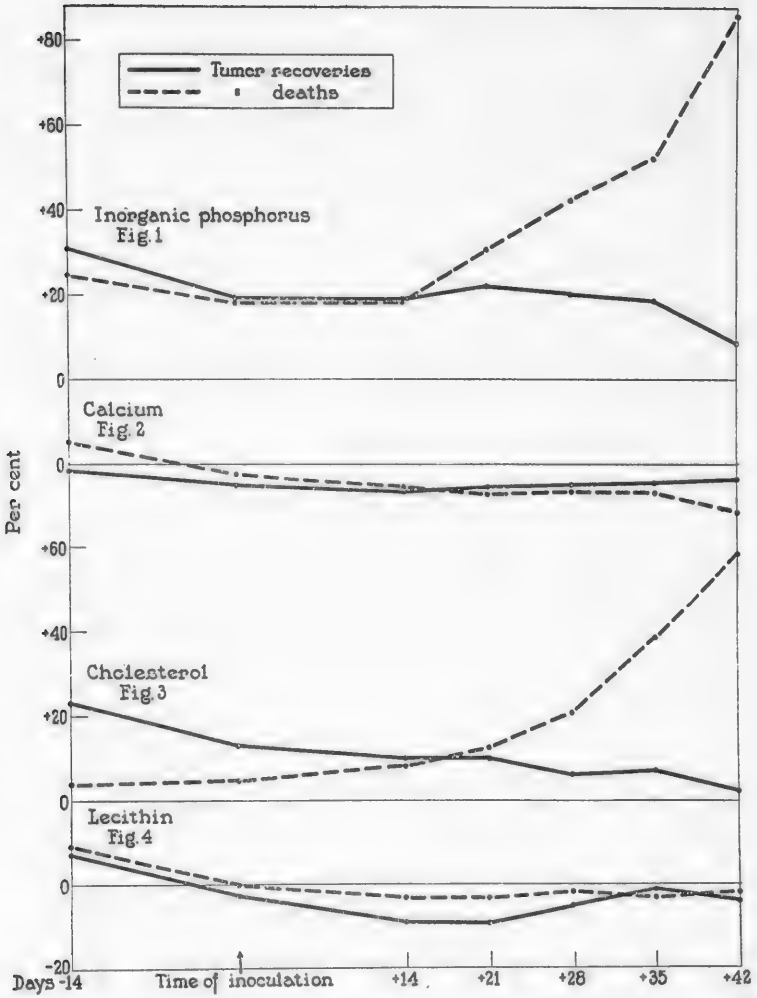
Group II. Tumor Recoveries

Days	Inorganic phosphorus			Calcium			Cholesterol			Lecithin		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
−14	6.13 ±0.11	0.67	10.92	15.4 ±0.29	1.85	12.01	71.6 ±1.90	11.93	16.62	127 ±4.27	26.87	21.16
Inoculated	5.29 ±0.10	0.65	12.29	15.0 ±0.15	0.96	6.40	64.1 ±1.45	9.13	14.24	114 ±4.79	30.16	26.46
+14	5.67 ±0.15	0.93	16.40	14.5 ±0.14	0.91	6.28	63.1 ±0.76	4.80	7.61	107 ±4.38	27.57	25.77
+21	5.75 ±0.09	0.54	9.39	15.0 ±0.18	1.07	7.13	65.3 ±1.29	7.86	12.04	105 ±3.11	19.02	18.11
+28	5.81 ±0.08	0.50	8.61	14.7 ±0.11	0.68	4.63	62.0 ±1.39	8.48	13.68	115 ±3.49	21.36	18.57
+35	5.68 ±0.16	0.95	16.73	15.2 ±0.26	1.57	10.33	63.8 ±1.04	6.33	9.92	120 ±2.89	17.59	14.66
+42	5.05 ±0.14	0.83	16.44	15.1 ±0.17	1.03	6.82	59.7 ±1.34	8.21	14.18	114 ±2.52	15.40	13.51

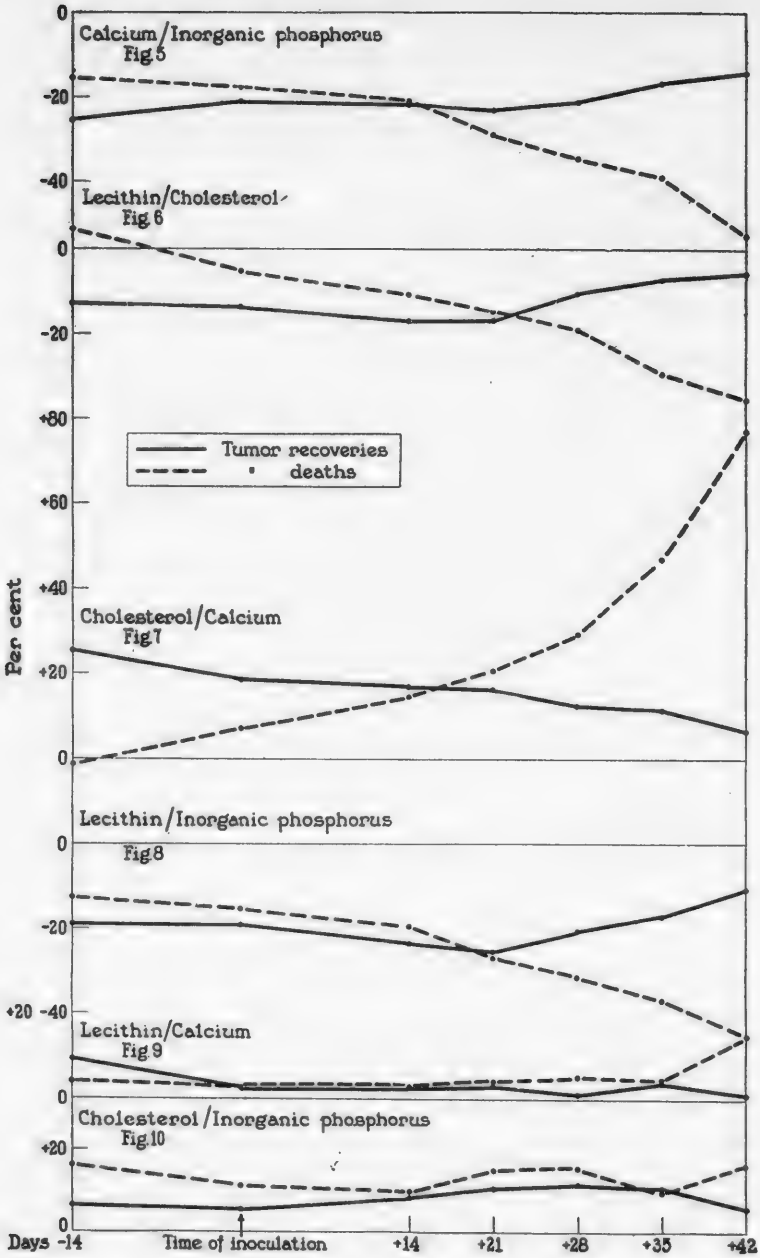
TABLE III

Coefficients of Correlation

	*r*P. Ca.	*r*P. Chol.	*r*P. Lec.	*r*Ca. Chol.	*r*Ca. Lec.	*r*Chol. Lec.	
Deaths from Tumor. Group I							
Trend	−0.654 ±0.12	+0.867 ±0.05	+0.165 ±0.21	−0.511 ±0.16	+0.290 ±0.20	−0.083 ±0.22	N = 7
Individual mean	−0.197 ±0.18	+0.653 ±0.12	−0.571 ±0.13	−0.200 ±0.18	+0.768 ±0.07	−0.066 ±0.20	N = 12
Series	−0.477 ±0.06	+0.537 ±0.05	−0.138 ±0.08	−0.319 ±0.07	+0.277 ±0.08	−0.072 ±0.09	N = 83
Recoveries from Tumor. Group II							
Trend	+0.113 ±0.21	+0.329 ±0.19	+0.337 ±0.19	+0.542 ±0.16	+0.729 ±0.11	+0.546 ±0.16	N = 7
Individual mean	−0.400 ±0.13	−0.428 ±0.13	−0.350 ±0.14	+0.171 ±0.15	+0.618 ±0.10	+0.066 ±0.22	N = 18
Series	−0.188 ±0.06	+0.206 ±0.05	−0.192 ±0.06	+0.086 ±0.07	+0.305 ±0.05	+0.029 ±0.07	N = 122


Tumor recoveries
deaths
Inorganic phosphorus
Fig. 1
Calcium
Fig. 2
Cholesterol
Fig. 3
Lecithin
Fig. 4
Per cent
+80
+60
+40
+20
0
0
+60
+40
+20
0
0
-20
Days -14
Time of inoculation
+14
+21
+28
+35
+42

FIGS. 1–4


Calcium/Inorganic phosphorus
Fig. 5
Lecithin/Cholesterol
Fig. 6
Tumor recoveries
deaths
Cholesterol/Calcium
Fig. 7
Lecithin/Inorganic phosphorus
Fig. 8
Lecithin/Calcium
Fig. 9
Cholesterol/Inorganic phosphorus
Fig. 10
Per cent
0
-20
-40
0
-20
+80
+60
+40
+20
0
0
-20
+20 -40
0
+20
0
Days -14
Time of inoculation
+14
+21
+28
+35
+42

FIGS. 5–10

DISCUSSION AND CONCLUSIONS

An examination of the results given in Tables I and II and the curves in Text-figs. 1 to 4 show certain striking differences between animals which died from the tumor and those that recovered. The differences were exhibited chiefly in the variation and trend of inorganic phosphorus, calcium, and cholesterol, the lecithin remaining more or less constant in both groups of animals.

Inorganic Phosphorus.—At the beginning of the experiment, the inorganic phosphorus (Text-fig. 1) in Group I was slightly lower than that obtained for Group II. At the time of inoculation both groups showed a decrease in inorganic phosphorus but the relative position was the same. The next examination, 14 days after inoculation, gave values of inorganic phosphorus which coincided. From this period, Group I gave increasing values for inorganic phosphorus and at the end of the experiment the results were 60 per cent higher than those recorded at the time of the first examination. Group II showed a steady decrease in inorganic phosphorus, the final level for this substance being 20 per cent lower than that obtained at the first examination. The inorganic phosphorus exhibited the highest per cent variation.

Calcium.—The calcium (Text-fig. 2) in Group I, at the −14 day period, was slightly higher than that found in Group II. With a gradual decrease in trend, this position was maintained until after the +14 day period at which time the position of the 2 groups was reversed. From this time, the results for Group I showed a more or less constant level until at the time of the last examination the values obtained for calcium were decidedly lower than those obtained for Group II. The calcium in the blood serum of Group II exhibited an uninterrupted increase beginning 14 days after inoculation and continuing throughout the remaining period of the experiment.

Cholesterol.—The cholesterol (Text-fig. 3) content of the whole blood in Group I was practically 20 per cent higher than that found in Group I at the −14 day examination. From this high level, the cholesterol in Group II showed a marked decrease which continued throughout the remainder of the experiment. At the

time of inoculation, the cholesterol content of the whole blood in Group I was practically the same as that of the preceding examination. From this time, however, the cholesterol increased rapidly, the last value being 55 per cent higher than that obtained for Group II.

Lecithin.—The lecithin (Text-fig. 4) level for Group I was slightly higher than that found in Group II throughout the entire experiment, with the exception of the results obtained at the +35 day period. At this time, the values for Group I were slightly lower than those for Group II. From the beginning of the experiment, both groups exhibited a gradual decrease in lecithin which continued until 14 days after inoculation. During the next 7 days both groups remained practically constant. From this period, Group II showed a gradual increase in lecithin, reaching a maximum on the +35 day examination. At the time of the last examination, however, the values were again lower than Group I. The lecithin for Group I throughout this same period maintained an almost constant level.

Ratios

The ratio curves (Text-figs. 5 to 10 inclusive) are presented in terms of per cent variation of the smoothed values from standard ratios (2).

The calcium-inorganic phosphorus ratio (Text-fig. 5) of Group I at the −14 day period was almost 10 per cent higher than that found in Group II. At the time of inoculation, the ratio for Group I showed a slight decrease while in Group II the ratio was markedly increased. From the time of inoculation throughout the remainder of the experiment, the ratio of calcium to inorganic phosphorus for Group I continued to decrease, the last determination being 36 per cent lower than that found in Group II. This low value was due to both an increase in inorganic phosphorus and a decrease in calcium. The trend of the ratio for Group II following inoculation showed a decrease of about 2 per cent at the +21 day period. From this time on, however, the ratio in this group continued to rise, reaching its highest value at the time of the last examination.

The lecithin-cholesterol ratio (Text-fig. 6) for Group I began with

a value about 4 per cent above the standard. From this time throughout the entire experiment, the ratio for Group I showed an uninterrupted decrease and at the last examination was 29 per cent below Group II, the decrease being affected chiefly by the marked increase in cholesterol occurring in this group of animals. At the —14 day examination, the lecithin to cholesterol ratio in Group II was some 16 per cent lower than that found in Group I. Throughout the next 28 day period, the ratio trend showed a slight decrease. During the next 7 days there was a tendency toward stabilization. Following this period of stabilization, however, the ratio trend in Group II showed a marked increase which continued throughout the remaining period of the experiment.

The cholesterol-calcium ratio (Text-fig. 7) for animals in Group I began with a value slightly under the standard. There occurred a gradual increase in the ratio up to the +14 day period. Following this, the ratio increased very rapidly and at the time of expiration the value for the cholesterol-calcium was 80 per cent higher than that found at the —14 day examination. This high ratio value was due to both an increase in cholesterol and a decrease in calcium. The cholesterol-calcium ratio for animals in Group II at the —14 day examination was 25 per cent higher than that found in the animals of Group I. From this initial value, the ratio trend continued to decrease gradually until the +21 day period. Following this date, the decrease which continued throughout the remainder of the experiment, was more rapid and at the final examination was practically 18 per cent lower than the first determination. A more detailed discussion of calcium-cholesterol relationship will be presented in a subsequent paper.

The trend of the lecithin-inorganic phosphorus ratio (Text-fig. 8) for both groups was somewhat similar to that of calcium-inorganic phosphorus. At the —14 day examination, Group I showed a ratio level about 5 per cent higher than Group II. With a slight decrease in trend, the 2 groups maintained practically the same relationship until the +21 day period when their ratio positions were reversed. From this date, the ratio for animals of Group I showed a rapid decrease while that for Group II gave increasing

values. This change in position for both groups of animals can be attributed chiefly to the variation of inorganic phosphorus.

The lecithin-calcium ratio (Text-fig. 9) for animals in Group I was slightly lower than that found in Group II. At the time of inoculation and throughout the following +14 day period, the ratios for both groups coincided. At the time of the +21 day examination, the ratio for Group I was slightly higher than that found in Group II. This position continued throughout the remainder of the experiment and at the time of the last examination the animals of Group I gave a ratio for lecithin-calcium 14 per cent higher than that of Group II.

The cholesterol-inorganic phosphorus ratio (Text-fig. 10) showed the least per cent variation. Group I which at the —14 day examination gave a ratio value 10 per cent higher than Group II, showed a gradual decrease in trend until the +14 day period. During the next 14 days a slight increase occurred to be followed by a rapid decrease. This decrease occurred at the +35 day examination and was the only time during the experiment when the cholesterol to inorganic phosphorus ratio for Group I was below that of Group II. This final examination resulted in a value for Group I which was 10 per cent higher than that found in Group II. During the 14 days preceding inoculation, the trend of the cholesterol-inorganic phosphorus ratio for Group II paralleled that of Group I. Following inoculation the ratio for Group II gradually increased, reaching its highest level on the +28 day examination. From this time, the ratio decreased, the decrease being more rapid throughout the last 7 days of the experiment.

It will be noted that in all ratio curves the change in position or trend occurred about 21 days after inoculation. This corresponds in a measure with the clinical course of the disease, for it is about this time that the ultimate fate of the animal is determined; from this point onward the tumor either pursues a malignant course or is gradually brought under control and recovery begins.

In the following table a comparison is made between the two groups of animals with respect to their ratio positions at the end of the experiment, 1 representing the highest and 4 the lowest value.

Ratio Positions of Tumor Animals

Group	Ca./P.	Lec./Chol.	Chol./Ca.	Lec./P.	Chol./P.	Lec./Ca.
II	1	1	4	1	4	1
I	4	4	1	4	1	1

When compared with a previous report on the influence of light environment (3), it will be noted that with the exception of the cholesterol to inorganic phosphorus ratio, the animals in Group I occupy a position similar to that of animals exposed to the ultra-violet light, while Group II occupies a position similar to animals living in total darkness.

Correlation

The coefficients of correlation for both groups of animals calculated on the basis of trend, individual mean values, and for each group series, are presented in Table III. It will be noted that the highest negative r inorganic phosphorus-calcium occurred in the animals of Group I. In Group II the r calcium-cholesterol were all positive while in Group I these same coefficients were all negative and of a somewhat greater magnitude. The r cholesterol-lecithin were all positive in Group II. These values while rather small were all negative in Group I.

Summarizing the results of this experiment, it will be noted that animals in which the tumor proved most malignant (Group I) there occurred a marked increase in inorganic phosphorus of the blood serum and cholesterol of the whole blood and a decrease in serum calcium. The ratios of cholesterol to calcium and lecithin to calcium increased in trend following inoculation, while the ratios of calcium to inorganic phosphorus, lecithin to cholesterol, and lecithin to inorganic phosphorus showed a marked decrease in trend. The animals in Group II exhibited trends and ratios of these 4 blood constituents similar to the control animals (2).

SUMMARY

Experiments are reported in which it was shown that the calcium, inorganic phosphorus, cholesterol, and lecithin in the blood of

normal rabbits were influenced by inoculation with a malignant tumor (4).

Animals in which death was due to tumor gave results on which the following conclusions are based:

1. A marked increase in inorganic phosphorus of blood serum and cholesterol of whole blood followed inoculation.

2. The ratios of cholesterol to calcium and lecithin to calcium increased in trend, while the calcium to inorganic phosphorus, lecithin to cholesterol, and lecithin to inorganic phosphorus ratios showed a marked decrease in trend.

3. The r cholesterol-lecithin were all negative.

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BLOOD AND BONE MARROW CELLS OF THE DOMESTIC FOWL

By CLAUDE E. FORKNER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

PLATE 6

(Received for publication, April 25, 1929)

The development of the supravital technique for the study of living cells has provided a new and interesting method for the cytological study of various animal tissues. This paper concerns the application of such methods to the blood and bone marrow cells of the domestic fowl. White Leghorn, Plymouth Rock and Black Jersey Giant roosters have been used in these experiments.

Direct Method of Counting

The problem of securing an accurate count of the white blood cells of the fowl has presented three difficulties. The avian red blood cells are nucleated, hence any method using the usual diluting fluids containing acetic acid will not permit discrimination between the red blood cell nuclei and the white blood cells. The second obstacle to an accurate count is the character of the thrombocytes. These structural elements in the fowl are about the size of lymphocytes and some of them may be confused either with red blood cells in which there is little hemoglobin, or with lymphocytes. The third factor is the rapidity with which coagulation occurs in the blood of the fowl.

Blain (1) has recently introduced a direct method for making total white blood cell counts on avian blood. He used two diluting fluids; Solution 1 contained neutral red 1:5000 made up in Locke's solution and adjusted to a pH of 7.4. The blood was first diluted with this solution at a temperature of 39°C., until the red cell pipette was one-half full. The mixture was shaken for 15 seconds, at the end of which time the pipette was filled with Solution 2, consisting of 12 per cent formalin in Locke's solution at pH 7.4. Shaking was then resumed for from 2 to 3 minutes. In this way he stated that every white cell had taken up sufficient neutral red to make possible its identification from the red blood cells. The latter took up no neutral red.

Prior to Blain's work the only method of estimating the number of white blood cells of avian blood was by indirect calculation, in which the total number of red and white corpuscles was determined in the counting chamber. Then, in films, the ratio between the number of red and white blood corpuscles was determined. From the total number of blood cells and from this ratio the number of white blood corpuscles per cubic millimeter may be calculated. This indirect method is subject to great error chiefly for two reasons. It presupposes an absolutely even distribution of red and white blood cells, which actually rarely obtains. The second source of error is in the thrombocytes. The size and character of these elements make their separation from lymphocytes in the counting chamber exceedingly difficult or impossible.

Blain (2) stated in a further communication that he identified no structures in avian blood corresponding to the platelets of mammals. It would appear, then, that the structures which other investigators, among them Ellermann (3), Albertoni and Mazzoni (4), Sugiyama (5), and which we ourselves consider to be thrombocytes have been included either in the red blood cell counts or in the white blood cell counts of Blain.

The method employed for the counting of the white blood cells in the studies reported in this paper has also made use of the vital staining properties of neutral red. A single diluting fluid has been devised, consisting of 25 mgm. of neutral red in 100 cc. of 0.9 per cent sodium chloride solution. This solution is filtered once and is kept at room temperature. It is preferable, but not essential to have the fluid warmed to body temperature at the time of use. The ordinary red blood cell pipette is used. The blood from a puncture of the wing vein or of the comb is drawn to the 0.5 mark and immediately diluted to the 101 mark, giving a dilution of 1:200. The pipette is shaken for 4 or 5 minutes as in ordinary blood cell counts. The counting chamber is filled in the usual manner. We have found it quite unnecessary to have the fluid at a definite pH or to introduce other complicated procedures.

It has been found that with this diluting fluid the polymorphonuclear elements and the monocytes stain in a characteristic manner, so that they can be recognized in the counting chamber with an 8 mm. lens and No. 10 objectives. The lymphocytes and thrombocytes are

not appreciably stained with the dye. The polymorphonuclear cells are characteristic in that the cytoplasmic border stains heavily and gives the appearance of a very dark, heavy rim to these cells. The monocytes take the dye more diffusely and do not have the deeply stained border. The lymphocytes and thrombocytes can be seen in the chamber but remain almost entirely unstained. Those red blood cells which have a small amount of, or no hemoglobin likewise fail to stain. The last three elements, lymphocytes, thrombocytes, and red blood cells without appreciable hemoglobin cannot be distinguished with any degree of accuracy in the counting chamber. The white blood cell count is made in the following manner: The total number of polymorphonuclear cells and monocytes is counted in the whole area of the ruled portion of the chamber (0.9 cu. mm.). This value taken together with the percentage of these combined elements obtained from the differential count permits a calculation of the total leucocyte count, exclusive of thrombocytes. An example will clarify this explanation.

Total number of polymorphonuclear cells and monocytes in 0.9 cu. mm.	7200 cells
Total number of polymorphonuclear cells and monocytes in 1 cu. mm.	8000 cells
Differential leucocyte count	
Polymorphonuclear eosinophiles	50 per cent
" neutrophiles	4
" basophiles	2
Monocytes	10
Total polymorphonuclear cells and monocytes	66 per cent
Small lymphocytes	30
Large "	4
	100 per cent

Therefore, 66% of total count = 8000 cells

$$100\% \text{ " " " } = \frac{8000}{66} \times 100 = 12{,}121+ \text{ white blood cells per cu. mm.}$$

By this method the difficulties of confusing the thrombocytes with the white blood cell count or with the erythrocyte count are overcome. There is also the advantage that the red blood cells may be counted in the same chamber as the leucocytes. This is practicable in the chicken because of the relatively low erythrocyte and relatively high leucocyte counts.

It should be stated here that the above method has been employed in this laboratory on many animals over a period of two years with con-

sistent results. The accuracy has also been tested by using the method on human blood, where entirely consistent results were obtained. The method was tried on a case of human lymphatic leukemia with a count of 60,000 cells. None of the lymphocytes contained any significant amount of dye, whereas the polymorphonuclear leucocytes and monocytes stained as they do in chickens' blood. It is pointed out that the method is readily adaptable for the counting of human blood, in which there are many normoblasts. I recall a case of sickle cell anemia in which there were 40,000 to 70,000 normoblasts per cubic millimeter of blood. The usual acetic acid diluting fluid does not distinguish these cells from leucocytes and thus a false leucocyte count was obtained. Likewise the normoblastic showers occurring in pernicious anemia are apt to lead to an erroneous white blood cell count unless a method of the type here outlined is resorted to.

Supravital Studies

Because of the diversity of opinion concerning the character of the cells in the blood of the domestic fowl, it seems necessary to give in some detail descriptions and illustrations of the types of cells encountered. It should be pointed out at this time that the following descriptions of cells apply only to the domestic fowl. Other kinds of birds, as pigeons, doves, etc., show a somewhat different picture.

Detailed observations on the blood and bone marrow cells of the chicken have been made by the use of the supravital technique and are reported in this communication. This is now a generally accepted method of great value to the cytologist for the study of living cells. It yields information concerning the motility, phagocytic power, mitochondrial content, fragility, and life history of the cells, which cannot be obtained by studying fixed smears or sections. The method has been used for many years by isolated investigators and has been more recently advocated and described by Simpson (6), Sabin (7), and others. For detailed descriptions of the technique, their papers should be consulted. In these studies neutral red and Janus green have been used as in the method given by Sabin (7).

Descriptions of Blood Cells

Red Blood Corpuscles (Fig. 11).—These cells in the chicken are all nucleated. They are oval in shape and considerably larger than the

red blood cells of man. The nucleus is usually slightly irregular and roughly oval in shape, and is smaller than that of a small lymphocyte. It is about the same size, or somewhat smaller than that of a thrombocyte. The nuclei of the normal red corpuscles are distinct from those of any other cells in that most of them are clear and show very little nuclear structure. Occasionally one finds a nucleus of a red blood corpuscle which is somewhat shrunken, more round than usual, and which has a more distinct, reticular structure. Around the nuclei of red blood corpuscles one almost invariably finds a few bluish-green, rod-shaped mitochondria which stain specifically with Janus green. The cytoplasm also contains from none to several small, reddish-brown bodies which are usually near the nucleus, but they often move about and may be far out near the cell border. In the supravital film, cells are sometimes found which obviously belong to the erythrocyte series, but in which there is no hemoglobin or a barely perceptible amount (Fig. 8). These cells have been confused with thrombocytes (Figs. 7, 9). However, careful scrutiny will always reveal an intact cellular membrane. The cytoplasm is much more abundant than that of the thrombocyte and the nucleus is distinct. These we have called degenerating forms of red blood cells. Clinging to their nuclei one can often see refractive vacuoles which are rarely stained and which are different from the stained vacuoles one sees in thrombocytes.

Thrombocytes (Figs. 7, 9).—These cells in the fowl are nucleated and are about the size of small or intermediate lymphocytes. They are distinctly smaller than erythrocytes. Their configuration is usually irregular in contrast to the round lymphoid cells and the oval red blood cells. The nuclei are distinguished by a very pronounced splotching which is so marked that it often appears vaculated. This is in contrast to the clear, almost structureless nuclei of the erythrocytes, and to the softer, more cloudy appearance of the nuclei of the lymphocytes. The cytoplasm of thrombocytes is often quite clear at first (Fig. 9), but after a few minutes' exposure to neutral red and Janus green, vacuoles (one or a few) develop at one or both ends of the nuclei (Fig. 7). These vacuoles stain a muddy brown color, are non-refractive, and increase in size with longer exposure to the dye. Sometimes a few very delicate, barely perceptible mitochondria appear in the perinuclear area. These bodies are much less constantly found and are not as large or as conspicuous as in lymphocytes.

Thrombocytes occur characteristically in clumps of two, three, or many cells, but are also found singly. In this respect they behave as do the blood platelets of mammals.

Polymorphonuclear Eosinophiles (Fig. 1).—These cells are numerous in the blood stream and, as has been shown by Moore (8) and others, are the elements which are specifically increased in fowl typhoid. They have a lobulated nucleus with a reticular chromatin network. The cytoplasm is filled with rod-shaped, specific granules which stain red with eosin in fixed preparations and stain a golden-yellow with neutral red in supravital films. These rods are not very uniform in size and shape. Some of them are short and almost round; others are sometimes club-shaped. The majority of them, however, are uniform in their size and staining reactions. After a few minutes' exposure to the dye, one or more vacuoles may appear in the cytoplasm. These vacuoles stain a reddish-orange color and increase in size with longer exposure to the dye. Mitochondria are rarely seen. When present, they are delicate filaments or dots staining specifically with Janus green. The polymorphonuclear eosinophiles are actively motile cells. They are probably comparable, in their function, to the polymorphonuclear neutrophiles of human blood.

Polymorphonuclear Cells with Pseudo-eosinophilic Granules (Fig. 2).—These elements have been commonly referred to in the literature as true eosinophiles, as eosinophiles with round granules, and as neutrophiles. They differ from the eosinophilic cells with rods not only in the shape of the granules, but also in their chemical reaction to the dye. The granules are really pseudo-eosinophilic. They take very little of the color in neutral red preparations. The granules are uniform in size and color and are quite small. They rarely fill the cytoplasmic area. The granules are very similar to the granules of amphophiles or neutrophiles of rabbits. The nucleus is usually bilobed. Mitochondria are commonly present among the specific granules. The cells are rarely as actively motile as the cells with eosinophilic rods.

Basophiles (Fig. 4).—The cells with specific basophilic granules are about the same size as those with eosinophilic rods. The nucleus is usually a single, irregular or round mass, but may be lobulated. The nucleus shows a diffuse chromatin distribution. The cytoplasm is filled with red specific granules. The cells are only slightly motile.

Monocytes (Fig. 5).—These are conspicuous cells in the chicken's blood. They vary somewhat in size from that of the polymorphonuclear cells to slightly larger. The nucleus is commonly indented or horseshoe-shaped but may be round or lobulated. Mitochondria are abundant, small, and may be in the shape of filaments, rods, or dots. The cytoplasm possesses a hazy appearance in contrast to the clear, glassy cytoplasm of lymphocytes. Neutral red bodies are prominent features and are often grouped together, forming a rosette in the bay of the nucleus. These neutral red bodies (segregation apparatus) increase in size with longer exposure to the dye. They stain a brick-red color and are almost all non-refractive. These cells are motile and have the same type of motility as has been described by Sabin (7) for the monocytes of human blood.

Lymphocytes (Figs. 6, 10).—Small, intermediate, and large lymphocytes are easily distinguished in fowl blood. The small lymphocytes are much more numerous than the larger forms. They are about the size of human small lymphocytes, but appear much smaller because of the larger red blood cells with which to compare them. They are similar in all respects to the lymphocytes of human blood. The nucleus is round or slightly indented and is relatively large in proportion to the amount of cytoplasm. The nuclear structure is made up of masses of chromatin which fade off gradually into the surrounding nuclear structure, giving a soft, cloud-like effect in contrast to the nuclear structure of thrombocytes and erythrocytes. In the cytoplasm of lymphocytes one finds large, coarse mitochondria as a constant feature. These Altmann bodies, which stain specifically with the Janus green, are apt to be perinuclear in their distribution and are sometimes grouped on one side of the nucleus. Many of the lymphocytes possess one or several small, red bodies in the cytoplasm. These structures stain a dark red in contrast to the brick-red of the monocyte granules and the brown of the thrombocyte segregation bodies. These neutral red bodies of the lymphocyte are round and are refractive, whereas the neutral red bodies of the monocytes and thrombocytes are non-refractive. The lymphocytes are only slightly motile in very fresh preparations. They move slowly with the nucleus near the front of the cell.

The accompanying table (Table I) gives the average total and per-

TABLE I

Blood Cell Counts of Normal Chickens

Fowl No.	Date	Total erythrocytes per cu. mm.	Total thrombocytes per cu. mm.	Total leucocytes per cu. mm.	Hemoglobin (Newcomer)	Eosinophiles with rods	Pseudoeosinophiles	Basophiles	Monocytes	Lymphocytes	Myelocytes	Unclassified	Total eosinophiles with rods per cu. mm.	Total pseudo-eosinophiles per cu. mm.	Total basophiles per cu. mm	Total monocytes per cu. mm.	Total lymphocytes per cu. mm.
						per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*					
55 B	11/ 2/27	3,270,000		17,670	65	31		10	14	42			6,008		1,767	2,474	7,421
	11/ 9/27	3,310,000	101,090	25,920	56	25	1	2	9	62		1	6,480	259	518	2,333	16,070
	11/12/27	3,220,000	46,137	11,830	60	26		3	10	60	1		3,076		355	1,183	7,098
	11/14/27	3,520,000	40,800	12,000	60	18	3	4	13	62			2,160	360	480	1,560	7,440
	11/19/27	3,020,000	17,961	8,553	65	32		6	8	54			2,737		513	684	4,619
	11/21/27	3,600,000	13,793	13,793	65	23	3	3	18	52		1	3,172	414	414	2,483	7,172
	11/26/27	2,960,000	23,716	26,351	70	31		6	23	40			8,169		1,581	6,061	10,540
	11/28/27	2,300,000	29,716	11,607	70	25	1	2	15	57			2,902	116	232	1,740	6,616
	12/ 1/27	3,540,000	39,130	13,043	70	19	1	3	8	69			2,478	130	390	1,043	9,000
	12/ 6/27	3,660,000	52,800	28,225	68	23	1	7	19	50			6,492	282	1,976	5,363	14,113
64 B	4/ 7/28	4,620,000	9,420	15,710	60	49	1	5	19	26			7,700	157	786	2,985	4,085
	4/11/28	3,700,000	17,976	29,960	60	43	1	10	9	37			12,882	300	3,000	2,700	11,085
51 B	10/20/27	2,810,000	35,156	9,375	60	37	3		7	53			3,468	281		656	4,970
54 B	11/ 2/27	3,070,000	47,823	17,390	57	33	8	5	5	48			5,739	1,391	870	870	8,347
65 B	4/ 7/28	3,760,000	23,280	23,280	75	66		1	13	20			15,365		233	3,026	4,656
	4/11/28	3,620,000	20,882	20,882	70	48	11	5	21	15			10,023	2,297	1,044	4,385	3,132
66 B	4/11/28	3,600,000	5,408	6,760	77	44	2	1	14	39			2,974	135	68	946	2,636
	4/16/28	3,390,000	25,025	22,750	72	24	2	5	8	61			5,460	455	1,137	1,820	13,878
1 C	10/19/28	3,750,000	10,672	26,686	46	48	1	1	17	32			12,809	267	267	4,536	8,540

	10/22/28:																
	11:45	2,810,000	52,382	33,795	52	33	2	3	34	28			11,152	676	1,014	11,490	9,463
	2:15	2,835,000	30,640	30,640	43	32		2	32	34			9,805		613	9,805	10,417
2 C	2:45	2,730,000	28,192	26,597		40	4	2	20	34			10,639	1,064	532	5,319	9,043
	3:15	3,030,000	34,513	33,508		31	2	7	23	37			10,387	670	2,345	7,708	12,398
	3:45	3,080,000	21,912	27,390		44		4	25	27			12,052		1,096	6,847	7,395
	4:30	2,830,000	42,543	39,030		30	3	7	30	29		1	11,709	1,171	2,732	11,709	11,319
	10/23/28	2,970,000	11,556	29,630	52	38		2	38	19		3	11,259		593	11,259	5,630
4 C	10/29/28	2,780,000	27,960	41,730	58	46		4	24	26			19,196		1,669	10,015	10,850
5 C	1/24/29	3,610,000	142,048	73,600	57	29	1	2	3	65			21,344	736	1,472	2,208	47,840
6 C	1/25/29	3,150,000	27,306	33,300	69	39		10	17	34			12,987		3,330	5,661	11,322
Average		3,267,000	34,990	24,586	62.9	34.72	1.76	4.21	17.1	41.79			8,642	385	1,069	4,444	9,900

centage values of each of the structural elements found in the blood of eleven domestic fowls. The total number of counts reported is 29. The average red blood cell count was 3,267,000 cells per cubic millimeter. The highest and lowest counts were 3,760,000 and 2,300,000 erythrocytes per cubic millimeter respectively. The average thrombocyte value was 34,990 cells per cubic millimeter, with a variation from 5,408 to 142,048 thrombocytes per cubic millimeter. Total leucocytes averaged 24,586 cells per cubic millimeter and varied between 6,760 and 73,600 cells per cubic millimeter. The hemoglobin (Newcomer method) varied from 46 per cent to 77 per cent. The average was 62.9 per cent.

Eosinophiles with rods varied between 18 and 66 per cent in different animals and averaged 34.72 per cent. Pseudo-eosinophiles with small, round granules were not encountered in ten of the counts. The highest percentage obtained was 11, and the average 1.76 per cent. Basophiles were found in all except one animal. The highest percentage value obtained was in Rooster 64, where they were 10 per cent. The average value was 4.21 per cent. Monocytes were found to be high in the chicken, varying from 3 to 38 per cent and averaging 17.1 per cent. Lymphocytes were usually more numerous than any of the white blood cells except thrombocytes. They also showed a wide variation between 15 and 69 per cent. The average value was 41.79 per cent. Myelocytes were rarely encountered and unclassified cells were few in number.

The total numbers per cubic millimeter of each of the types of leucocytes are given in Table I. Their average values obtained were:

	per cubic millimeter
Eosinophiles with rods	8,642
Pseudo-cosinophiles	385
Basophiles	1,069
Monocytes	4,444
Lymphocytes	9,900

Discussion of Blood Cells

The literature records the observation of numerous investigators on the blood of the fowl. Some of the more important of these studies have been summarized in Table II.

TABLE II

Blood Counts of Domestic Fowls as Recorded in Literature

Authors	Erythrocytes per cu. mm.	Leucocytes per cu. mm.	Thrombocytes per cu. mm.	Eosinophiles with rods	Pseudoeosinophiles	Basophiles	Monocytes	Lymphocytes		Hemoglobin	Degenerating types	Unclassified	Clasmatocytes
								Large	Small				
				per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Stöltzing (9)	3,860,000												
Malassez (10)	3,100,000												
Hayem (11)	2,400,000	26,300											
Moore (12)	3,637,000	20,081											
Ward (13)	3,283,000	36,185											
Mack (14)	3,017,000	55,272								87.3			
Albertoni and Mazzoni (4)	2,460,000	32,300	45,566										
Klieneberger and Carl (15)	3,117,000	35,000 to 60,000	22,900 to 130,000	29.5	4.5	2.2	None	63.8		62.0			
Blain (2)		18,630	None	49.4	8.7	3.6	5.7	32.8					
Warthin (16)	2,000,000 to 3,000,000	12,000 to 29,000		21.5	10.0	2.0	None	35.5	14.5		16.5		
Burnett (17)	3,324,000	17,921		28.8	3.3	4.3	5.5	58.0		76.0			
Schmeisser (18)	2,500,000 to 4,500,000	20,000 to 80,000		29.6	4.3	2.2	19.4	42.3		45.0 to 75.0 (Sahli)		2.2	
Breusch (19)	3,468,000	33,300		17.7	4.1	2.5	9.2	66.5					
This study (average values)	**3,267,000**	**24,586**	**34,990**	**34.7**	**1.8**	**4.2**	**17.0**	**41.8**		**62.9 (Newcomer)**	**Rare**	**Rare**	**Rare**

It will be seen that Mack (14) records 55,272 leucocytes per cubic millimeter as the normal count, whereas Blain's (2) and Burnett's (17) data show 18,000 cells per cubic millimeter as the average normal value. Likewise the thrombocytes have been variously recorded. Most of the investigators have failed to enumerate them. Klieneberger and Carl (15) state that they vary from 22,000 to 130,000 per cubic millimeter. On the other hand, Blain (2) has failed to find any of these structures. The percentages of the types of leucocytes have yielded markedly different figures in the hands of different investigators. Eosinophiles with rods are reported by Breusch (19) as constituting 17.7 per cent of the total leucocytes, whereas Blain (2) found 49.4 per cent of these cells. Klieneberger and Carl (15) identified no monocytes or transitional cells in normal domestic fowls. On the other hand, Schmeisser (18) states that there are 19.4 per cent of these elements in the blood.

An analysis of the literature reveals four causes for most of the discrepancies. These causes are: study of insufficient numbers of animals, failure to make a sufficient number of observations on single animals, lack of an adequate method of counting white blood cells, and confusion of thrombocytes with other elements of the blood. It must also be borne in mind that there are large individual fluctuations of the leucocyte counts of fowls which are considerably greater than those encountered in mammals.

Still another factor which has led to confusion in the interpretation of blood cells of the fowl is the method of classification of the granular leucocytes.

Niegolewsky (20), for example, described two types of oxyphil granular cells, one with large refractive granules and another with fine oxyphil granules. The former were numerous and the latter few in number. In addition he found two types of basophiles and also neutrophilic leucocytes. The latter had round or polymorphous nuclei and fine granules. Grünberg (21) described two types of eosinophilic leucocytes: (a) cells with crystalloid granules, and (b) cells with globular-like granules. These latter he stated were similar to the polymorphonuclear cells of man. Hirschfeld-Kaszmann (22) stated that three types of eosinophilic granular cells occurred: (a) cells with segmented nuclei and crystalloid granules, (b) cells with single nuclei and pale rod-shaped granules, and (c) cells similar to (b) but having round granules. They found no neutrophilic granular cells. Burnett (17) did not call the polymorphonuclear cells with rod or spindle-shaped granules eosinophiles. He believed that these cells resembled in their staining reaction and biological properties the polymorphonuclear neutrophiles of mammals. He found eosinophilic leucocytes with round granules which were similar to the eosinophiles of mammals. Kasarinoff (23) described the same types

of eosinophiles as did Grünberg (21). Klieneberger and Carl (15) found two types of eosinophiles. Those with rod-shaped granules they called pseudo-eosinophiles and those with small granules they labelled as eosinophiles with small granules.

Descriptions of Bone Marrow Cells

The method which has been applied in these studies to the bone marrow has been a modification of that employed for blood and is the same as that which has been used by Sabin and Doan (24) and others for the study of bone marrow, lymph nodes, and other tissues of animals. Neutral red and Janus green films are prepared as for making supravital studies on blood, except that the concentrations of the dyes are increased about three fold. Small pieces of representative bone marrow are then removed from an anesthetized or freshly killed animal. A suspension is made on the prepared slide by mixing the small piece of marrow in a drop of normal saline, or better yet, with the animal's own serum. This material is spread out on the slide over an area the size of the coverslip. The coverglass is then allowed to fall gently on the prepared marrow. The preparation is quickly rimmed with vaseline or melted paraffin and can be studied immediately in a constant temperature box at about 38 or 39°C. Material prepared in this way gives a fairly uniform distribution of the cells and a thin enough film so that the individual cells can be studied and counted. The cells survive for hours if kept at body temperature.

A complete review of the histology of the bone marrow has recently been written by Sabin (25). For this reason it is unnecessary to review again the literature on this organ.

The types of cells found in the bone marrow of the chicken are, in addition to the fully matured formed elements of the blood, immature forms of all the leucocytes and red blood corpuscles. The relative numbers of the various types of cells are recorded in Table III. In this table the numerical values are given of the cells found in the bone marrow of four normal, Plymouth Rock roosters, about 8 months of age.

The cells present in largest numbers in films of the bone marrow are mature red blood corpuscles. They constitute an average of 53.8 per cent of all the cells present. Mature polymorphonuclear eosinophiles, on the other hand, constitute only 3.9 per cent of all the cells and 8.18

TABLE III

Survey of Cells of Bone Marrow of the Domestic Fowl

Rooster No.	Marrows examined	Total number of cells counted			Mature R. B. C. Per cent of all cells	Eosinophiles								Neutrophiles			
		Mature		All other cells		Mature P. M. E.		Myelocytes "A"		Myelocytes "B"		Myelocytes "C"		Mature P. M. N.		Myelocytes	
		R. B. C.	P. M. E.			Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.
2 C	Rt. femur Lt. " Rt. tibia	769	80	798	46.86	4.85	9.11	0.48	0.91	2.31	4.33	8.74	16.40			0.48	0.91
1 C	Rt. femur Lt. " Rt. tibia Lt. "	839	49	375	66.43	2.69	8.02			0.95	2.83	8.16	24.29			0.24	0.71
4 C	Rt. femur Rt. tibia Lt. " Rt. radius	828	12	660	55.20	0.80	1.79	0.47	1.04	2.20	4.91	9.93	22.17			0.13	0.29
5 C	Rt. femur Lt. " Rt. tibia Lt. "	876	138	862	46.70	7.41	13.80	0.75	1.40	1.17	2.20	11.30	21.2	0.16	0.30	0.21	0.40
Average.....		828	70	674	53.8	3.94	8.18	0.42	0.84	1.66	3.57	9.50	21.02	0.04	0.08	0.26	0.58

TABLE III—*Concluded*

Rooster No.	Marrows examined	Basophiles		Monocytes		Small, round cells, probably lymphocytes		Undifferentiated cells—megalo-, mono-, lympho-, myeloblasts		Erythroblasts		Clasmatocytes		Osteoclasts		Unclassified	
		Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.
2 C	Rt. femur Lt. " Rt. tibia	0.31	0.58	0.67	1.25	13.78	25.85	4.37	8.09	16.15	30.29	1.09	2.05			0.12	0.23
1 C	Rt. femur Lt. " Rt. tibia Lt. "					6.65	19.81	7.67	22.87	7.05	21.00					0.16	0.47
4 C	Rt. femur Rt. tibia Lt. " Rt. radius			5.93	13.24	10.93	24.40	2.74	6.11	10.13	22.62	0.48	1.64	0.13	0.30	0.67	1.49
5 C	Rt. femur Lt. " Rt. tibia Lt. "	0.05	0.10	3.09	5.80	14.71	27.60	3.04	5.70	11.46	21.5						
Average.....		0.09	0.17	2.42	5.07	11.52	24.41	4.46	10.69	11.20	23.85	0.39	0.92	0.03	0.07	0.24	0.55

per cent of all the cells, exclusive of mature red blood corpuscles. The accompanying table has been arranged to give the percentage value of each constituent element both in relation to the total of all the cells present and in relation to the number of cells, exclusive of mature red blood corpuscles. The reason for this is that most of the mature erythrocytes probably represent extravasations from traumatic hemorrhage and are not true parenchymal elements of the marrow.

Myelocytes (Figs. 14, 15, 16).—In this paper the myelocytes have been grouped arbitrarily into A, B, and C classes, according to the plan employed by Sabin, Austrian, Cunningham, and Doan (26). Myelocytes A are of the earliest type and represent the next stage of development after the myeloblast. Myelocytes A include cells with 1 to 10 specific granules in the cytoplasm. Myelocytes B (Figs. 14, 15) have more specific granules than Myelocytes A, but do not possess their full quota. Myelocytes C (Fig. 16) represent the next stage of development in which there is the full quota of specific granules in the cytoplasm, but the cells still retain their mononuclear characteristics.

The myelocytes of the chicken's blood are not only distinguished by the number of their specific granules, but also by their character. The granules are almost always round or globular, whereas the fully mature cell possesses elongated, rod-shaped granules. Moreover, the younger the specific granules are the more intensely they stain with neutral red. The mature granules of the adult polymorphonuclear cell and of the very late myelocytes stain a yellowish copper color with neutral red. The earlier myelocyte granules (Fig. 15) are darker in color, staining a brownish red. Mitochondria are readily seen in the early myelocytes. They are scattered through the cytoplasm and are delicate rods or dots which stain specifically with Janus green. With greater accumulation of specific granules, the mitochondria become less conspicuous and sometimes cannot be seen. Myelocytes A are are few in number in normal bone marrow. The average value was slightly less than 1 per cent of the cells, exclusive of mature erythrocytes. Myelocytes B composed 3.57 per cent. These cells were present in about the same relative proportions as Sabin and Doan (24) found them in normal rabbit bone marrow. Myelocytes C were represented by 21.02 per cent of the cells, distinctly less than their proportion in rabbit bone marrow.

Pseudo-eosinophilic myelocytes and polymorphonuclear pseudo-eosinophiles were only occasionally found in the bone marrow, where they constituted one-half of 1 per cent of the cells. They were identical with the pseudo-eosinophilic cells of the blood.

Basophiles.—These cells were likewise rare in the bone marrow. They, too, were identical with the basophilic cells which were found in the blood and have been described in an earlier part of this paper. In two of the animals no basophiles were seen in the marrow films. The average value for basophiles in the bone marrow was less than 0.2 per cent. Sabin and Doan (24) found in rabbits that they constituted from 0.29 to 2.02 per cent of the bone marrow cells.

Monocytes.—About 5 per cent of the parenchymal elements of the marrow are monocytes. They are identified by the criteria previously given in the section on blood cells, and need no further description here. It is not difficult to distinguish them from the early myelocytes because of the larger and more refractive granules of the latter.

Lymphocytes.—Contrary to the findings in bone marrow of mammals, there is a considerable number of small round cells (Fig. 3) in every film of bone marrow equivalent to about 25 per cent of the cells. They are probably small lymphocytes. It is often difficult in the marrow to differentiate these elements from primitive cells and thrombocytes. The characteristics which render the distinction easy in a thin film of blood are often obscured by the crowding of the cells and lack of perfectly uniform staining.

Undifferentiated Cells (Fig. 13).—The early undifferentiated cells of the bone marrow I have chosen to group together under one heading for the reason that I have not been able to say in numerous instances whether given cells were megaloblasts, monoblasts, lymphoblasts, myeloblasts, or primitive cells. The problem of sub-classifying these "blast" cells in the marrow of the chicken is much more difficult than to recognize and classify them when they occur in the blood. The reasons are obvious. The staining is not as uniform, the cells are more crowded, and one cannot have as an aid the character of the other accompanying cells, which aid is often of great value in a study of blood films. In the bone marrow, spleen, and lymph nodes of mammals it is usually not difficult to segregate the cells into the various types of blasts, but I do not believe that there is sufficient evidence in the

chicken for such a rigid sub-division of the undifferentiated marrow cells. This group of blast cells represents about 10.5 per cent of the parenchymal elements. They are about the size of myelocytes. The nucleus may be central or peripheral and frequently contains a large nucleolus. The cytoplasm usually contains no stainable material in supravital films.

Erythroblasts (Fig. 12).—Immature red blood cells are separated from the mature red corpuscles by their larger and more conspicuous nuclei, by the presence of more neutral red bodies in the cytoplasm, and often by a decreased amount of hemoglobin. The immature red corpuscles are frequently less oval and more round than the adult forms. The total number of erythroblasts represents about 24 per cent of the cells, exclusive of mature red blood cells, and about 11 per cent of the total cells present.

Clasmatocytes (*Macrophages*).—The characteristic by which these cells are recognized is dependent largely on their phagocytic properties. They engulf leucocytes, red corpuscles, and débris. The vacuoles of the living cells stain brilliantly with various shades of red, yellow, and brown. These vacuoles increase in size with longer exposure to the dye, and are not arranged in any definite pattern. The nucleus is usually round and placed near the center of the cell. The shape of macrophages is often irregular. They vary in size, but are commonly about the size of monocytes or other large leucocytes. These cells constitute about 1 per cent of the marrow elements.

Osteoclasts (Fig. 17).—The surveys of the bone marrows of four chickens reported in this communication show osteoclasts to be present in only one. In this animal they represented 0.3 per cent of the marrow cells. The ends of the long bones often contain many spicules of bone, and in these regions osteoclasts are more numerous than in the shafts. They are very large, multinucleated cells. The nuclei do not appear to have any definite pattern, but are of about equal size and scattered through the cytoplasm. The cytoplasm often contains considerable numbers of vacuoles or granules which are quite homogeneously distributed throughout the cytoplasm and may tend to obscure the nuclei. These vacuoles stain brown with neutral red.

Thrombocytes have not been observed in the marrow of the long

bones of these chickens. Their origin is obscure and work is now in progress to determine more about the life history of these elements.

SUMMARY AND CONCLUSIONS

1. A simple, direct method of counting leucocytes of the fowl is described.

2. Twenty-nine complete, morphological studies of the blood of eleven domestic fowls are recorded.

3. The characteristics of the cells found in the blood and bone marrow are described in detail and their relative numbers reported.

4. The supravital technique, using neutral red and Janus green, enables one to separate and classify accurately the confusing cells of the blood and bone marrow.

5. These studies provide a basis for future experimental studies on the blood and bone marrow cells of the fowl.

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EXPLANATION OF PLATE 6

FIG. 1. Typical polymorphonuclear eosinophile with rods from peripheral blood. Stained with neutral red.

FIG. 2. Pseudo-eosinophile from peripheral blood of domestic fowl. This cell is slightly larger than the typical pseudo-eosinophile. Stained with neutral red and Janus green.

FIG. 3. Small, round cell of bone marrow of the domestic fowl, probably a lymphocyte.

FIG. 4. Basophilic leucocyte of the peripheral blood. Note that the granules vary somewhat in their affinity for neutral red.

FIG. 5. Monocyte of the peripheral blood. Mitochondria stained with Janus green and segregation bodies stained with neutral red. Note the tendency for grouping of neutral red bodies about the centrosphere, a common, but not invariable characteristic of these cells.

FIG. 6. Large lymphocyte from peripheral blood. Stained with neutral red and Janus green. There were no neutral red bodies in this cell, a fairly common finding. Mitochondria are large.

FIG. 7. Two thrombocytes grouped together in the peripheral blood. No mitochondria were seen in these cells, but there were numerous neutral red vacuoles. Stained with neutral red and Janus green.

FIG. 8. A degenerating red blood corpuscle of peripheral blood. Note the absence of hemoglobin, but the preservation of the cellular membrane. A few refractive, unstained bodies are near or on the nucleus. These cells have been confused with thrombocytes. Compare with Figs. 7 and 9.

FIG. 9. Two thrombocytes of peripheral blood. Mitochondria are more conspicuous than in the usual thrombocyte. A neutral red vacuole has begun to develop. Stained with neutral red and Janus green.

FIG. 10. Intermediate lymphocyte of peripheral blood. Slightly larger than a small lymphocyte. Stained with neutral red and Janus green. Two small neutral red bodies are present.

FIG. 11. Typical erythrocyte of peripheral blood.

FIG. 12. Erythroblast from the bone marrow. Note the smaller amount of hemoglobin than in the mature erythrocyte.

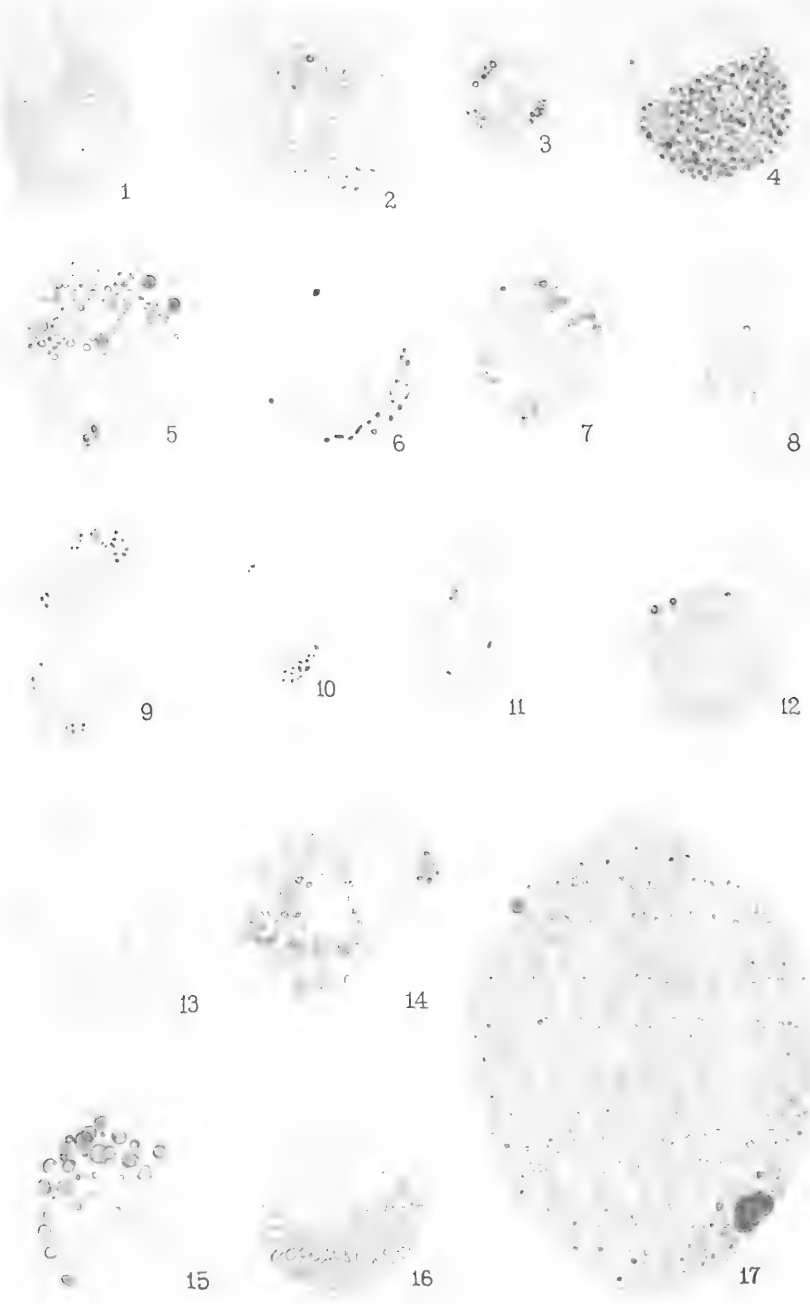
FIG. 13. An undifferentiated cell of the bone marrow. This cell has an eccentric nucleus. Many of them have nuclei in the center of the cell. Neutral red and Janus green stain.

FIG. 14. A myelocyte "B" of the bone marrow. Specific granules are refractive and round. Stained with neutral red. Mitochondria are often abundant, but are not shown in this drawing.

FIG. 15. Myelocyte "B" of a somewhat earlier stage than that shown in Fig. 14. Specific granules vary considerably in size and take a somewhat deeper color than in the later myelocytes. Stained with neutral red.

FIG. 16. Myelocyte "C" of bone marrow. Some of the specific granules are beginning to assume the color and shape of those of the fully mature polymorphonuclear eosinophile shown in Fig. 1. Neutral red stain.

FIG. 17. Osteoclast of the bone marrow. Nuclei are round and almost obscured by the neutral red bodies.



(Forkner: Blood and bone marrow cells of domestic fowl)

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